

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Tapas Mukhopadhyay, *et al.*

Serial No.: 10/043,877

Filed: January 9, 2002

For: ANTIHELMINTHIC DRUGS AS A
TREATMENT FOR
HYPERPROLIFERATIVE DISEASES

Group Art Unit: 1642

Examiner: Brandon J. Fetterolf

Atty. Dkt. No.: INRP:095US

CERTIFICATE OF ELECTRONIC SUBMISSION

DATE OF FILING December 8, 2006

APPEAL BRIEF

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APPEAL BRIEF

MS Appeal Brief

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellants hereby submit this Appeal Brief to the Board of Patent Appeals and Interferences in response to the Office Action dated July 10, 2006. A Notice of Appeal is submitted concurrently herewith. A Request for an Extension of Time for two months is also enclosed. With this extension, the deadline for submission of this Brief is Monday, December 11, 2006, by virtue of the fact that December 10, 2006, falls on a Sunday. A Request for Oral Hearing is being filed concurrently with this Appeal Brief.

A check for fees in the amount of \$1,450.00 for the Appeal Brief (\$500.00), Notice of Appeal (\$500.00) and a Two-Month Extension of Time (\$450.00) is enclosed. Should any additional fee(s) be required, consider this paragraph such a request and authorization to withdraw the appropriate fee under 37 C.F.R. §§ 1.16 to 1.21 from Fulbright & Jaworski L.L.P. Account No.: 50-1212/INRP:095US.

I. REAL PARTY IN INTEREST

The real parties in interest are the assignee, The Board of Regents, The University of Texas System and the exclusive licensee, Introgen Therapeutics, Inc.

II. RELATED APPEALS AND INTERFERENCES

No related appeals or interferences are presently pending.

III. STATUS OF THE CLAIMS

Claims 1-182 were originally filed January 9, 2002.

In a Response to Restriction Requirement dated March 29, 2004, Appellants filed an amendment in which claims 4-8, 11, 30-74, 78-82, 107-160, 163, 166, 168 and 171-175 were withdrawn from consideration as being drawn to a non-elected invention and/or species and claims 1-29, 75-106, 161-162, 164-167 and 169-182 were elected for prosecution.

Claims 1-3, 9-10, 12-29, 75-77, 83-106, 161-162, 164-165, 167, 169-170 and 176-182 were rejected in the Office Action dated June 28, 2004.

In a Response to Office Action dated October 28, 2004, Appellants filed an amendment in which claims 164-165, 167, 169-170 and 176-182 were canceled without prejudice or disclaimer, and in which 22, 100, 161 and 162 were amended.

Claims 1-3, 9-10, 12-29, 75-77, 83-106, 161-162 and 164 were rejected in the Office Action dated March 18, 2005.

In a Response to Office Action dated July 18, 2005, Appellants filed an amendment in which claims 161-162 were amended and new claims 183-184 were added.

Claims 1-3, 9, 12-19, 21-29, 75-77, 83-97, 99-106, 161-162 and 183-184 were rejected in the Office Action dated November 16, 2005.

In a Response to Office Action dated April 17, 2006, Appellants filed an amendment in which claims 2, 10-11, 13, 15, 17-22, 29-30, 32, 64, 76-86, 88, 90-100, 107 and 109 were amended, and in which claims 1, 9, 12, 64, 75, 161-162, 164-165, 167, 169-170 and 176-184 were canceled without prejudice or disclaimer.

Claims 2-8, 10-11, 13-63, 65-74, 76-160, 163, 166, 168, 171-175 were pending at the time of the Office Action dated July 10, 2006, with claims 4-8, 11, 30-63, 65-74, 78-82, 107-160, 163, 166, 168 and 171-175 withdrawn and claims 2-3, 10, 13-19, 21-29, 76-77, 83-97 and 99-106 standing rejected. Thus, claims 2-3, 10, 13-19, 21-29, 76-77, 83-97 and 99-106 are on appeal and the subject of this appeal brief.

IV. STATUS OF AMENDMENTS

No amendments have been filed subsequent to the final rejection.

V. SUMMARY OF CLAIMED SUBJECT MATTER

In one general aspect, as recited in claim 22, the present invention concerns a method for inducing apoptosis in a tumor cell expressing a tumor suppressor gene, by determining the status of the tumor suppressor gene status of the tumor cell and administering an effective amount of a benzimidazole to the tumor cell, wherein expression of the tumor suppressor gene by the tumor cell and benzimidazole results in the apoptosis of the tumor cell. *See, e.g.*, p. 8, lines 19-23; p. 15, lines 19-28; and Example 2 at p. 56, line 13 through p. 64, line 12.

In another general aspect, as recited in claim 100, the present invention concerns a method for treating a patient having cancer, wherein cancer cells express a tumor

suppressor, by determining the tumor suppressor gene status of the cancer cells and administering an effective amount of a benzimidazole to the patient where the expression of the tumor suppressor gene by the cancer cell and the administration of the benzimidazole results in the inhibition of the cancer. *See, e.g.*, p. 4, lines 28-31, p. 5, lines 15-18; and p. 14, lines 24-27.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1) Are claims 76, 83-97 and 99-106 properly rejected as being unpatentable over Camden (U.S. Patent No. 6,262,093, “Camden I”) in view of Perdomo *et al.*, *J. Cancer Res. Clin. Oncol.*, 124:10-18, 1998 (“Perdomo”) under 35 U.S.C. § 103(a)?

2) Are claims 76-77, 83-97 and 99-106 properly rejected as being unpatentable over Camden I in view of Perdomo in further view of Delatour *et al.*, *Therapie*, 31:505-515, 1976 (“Delatour”) under 35 U.S.C. § 103(a)?

3) Are claims 2, 10, 15-19, 21-29, 76, 83, 85, 88-97 and 100-106 properly rejected as being unpatentable over Camden (U.S. Pat. No. 5,880,144, “Camden II”) as evidenced by Camden I in view of Perdomo under 35 U.S.C. § 103(a)?

4) Are claims 3 and 77 properly rejected as being unpatentable over Camden II in view of Perdomo in further view of either Delatour or Nasr *et al.*, *J. Pharm. Sci.*, 74:831-836, 1985 (“Nasr”) under 35 U.S.C. § 103(a)?

5) Are claims 13-14 and 86-87 properly rejected as being unpatentable over Camden II in view of Perdomo and further in view of Lucci *et al.*, *Cancer*, 86:300-311, 2000 (“Lucci”) under 35 U.S.C. § 103(a)?

VII. ARGUMENT

A. Substantial Evidence Required to Uphold the Examiner's Position

The Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by “substantial evidence” within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *Gartside*, the Federal Circuit stated that “the ‘substantial evidence’ standard asks whether a reasonable fact finder could have arrived at the agency’s decision.” *Id.* at 1312. *See also Matsushita Elec. Indus. Co. v. United States*, 750 F.3d 927, 933 (Fed. Cir. 1984) (a decision is supported by substantial evidence when “a reasonable mind might accept [it] as adequate to support a conclusion.”). Accordingly, it necessarily follows that an Examiner’s position on appeal must be supported by “substantial evidence” within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Claims 76, 83-97 and 99-106 Are Not Rendered Obvious Over Camden I in View of Perdomo Under Under 35 U.S.C. § 103(a)

1. Camden I is Not Available As Prior Art

The Examiner has not met the burden of proof to establish a *prima facie* case of obviousness of claims 76, 83-97 and 99-106 over the combination of Camden I (Exhibit 1) and Perdomo (Exhibit 2) under 35 U.S.C. § 103(a). Simply put, Camden I is not available as prior art under 35 U.S.C. § 102(e) as Appellants have demonstrated conception and reduction to practice prior to the filing date of Camden I. This was demonstrated in the Second Declaration of Tapas Mukhopadhyay, Sunil Chada, Abner Mhashilkar and Jack A. Roth Under 37 C.F.R. § 1.131. (Exhibit 3). In the November 16, 2005 Office Action (Exhibit 4), the Examiner accepted this reduction to practice as it pertains to *in vitro* claims, stating that the “Declaration filed on 08/22/2005 under 37

CFR § 1.131 is sufficient to overcome the Camden et al reference with respect to claims 1-3, 9-10 and 12-29 as specifically drawn to a method of inducing apoptosis in a cell expressing a tumor suppressor gene comprising administering an effective amount of a benzimidazole to said cell, wherein the expression of the tumor suppressor gene by the cell and the benzimidazole results in the apoptosis of the cell.” November 16, 2005 Office Action, p. 2, para. 2 (Exhibit 4).

In response to the Examiner’s contention that the Second Declaration of Tapas Mukhopadhyay, Sunil Chada, Abner Mhashilkar and Jack A. Roth Under 37 C.F.R. § 1.131 was sufficient only to overcome *in vitro* claims, Appellants submitted the Third Declaration of Tapas Mukhopadhyay, Sunil Chada, Abner Mhashilkar and Jack A. Roth Under 37 C.F.R. § 1.131 (Exhibit 5). In this declaration, Appellants demonstrated a showing of facts of such character and weight as to establish conception prior to March 9, 1999, coupled with diligence in the reduction to practice of *in vivo* treatment of cancer.

The Examiner, instead of responding directly to the issue of the establishment of *in vivo* conception plus reduction to practice, argues that the “Camden reference is a U.S. Patent or U.S. Patent application publication of a pending or patented application that claims the rejected invention or an obvious variant.” July 10, 2006 Action, p. 2 (Exhibit 6). Thus, it appears that the Examiner is contending that the Third Declaration of Tapas Mukhopadhyay, Sunil Chada, Abner Mhashilkar and Jack A. Roth under 37 C.F.R. § 1.131 is inappropriate “when the reference is claiming the same patentable invention or are obvious variants.” *Id.*, p. 2.

Under current interference rules as well as controlling case law, the standard by which the Examiner’s position would be proper is “two-way” unpatentability: “[a]n

interference exists if the subject matter of a claim of one party would, if prior art, have anticipated or rendered obvious the subject matter of a claim of the opposing party **and vice versa.**” 37 C.F.R. § 41.203(a) (emphasis added); *Winter v. Fujita*, 53 USPQ2d 1234, 1243 (BPAI 1999). Thus, the claims at issue would need to be drawn to the same patentable subject matter as the Camden I claims, *i.e.*, to **interfere** with the claims of those references. Appellants contend that this is not the case.

Anticipation, in this context, requires that a patent claim recite every limitation of the presently claimed invention, either explicitly or inherently. *See In re Schreiber*, 128 F.3d 1473, 44 USPQ2d 1429, 1432 (Fed. Cir. 1997). Further, to establish a *prima facie* case of obviousness, the claim must: (1) teach or suggest all the claim limitations; (2) provide some suggestion or motivation to modify the reference; and (3) provide a reasonable expectation of success. MPEP § 2142; *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

a) The Examiner has Failed to Set Forth the Basis for Failing to Consider the Third Declaration with Particularity

The Examiner has failed to clearly set forth any analysis as to why he believes that the Third Declaration is not appropriate under 37 C.F.R. § 1.131. Further, there is **no** evidence of record to support the Examiner’s conclusion regarding the interfering nature of the pending claims as compared to those of the Camden I reference.

The Examiner is required to set clearly forth reasons for any rejection. *See* 37 C.F.R. § 1.104(c)(2). Appellants fail to discern any clear analysis explaining why the invention of Camden I is the same as that of the present invention, or why the present invention is an obvious variant of Camden I. Indeed, there is **no** evidence of record to support the Examiner’s conclusion. If the Examiner is relying on facts within his own

personal knowledge in setting forth this rejection, Appellants, in accordance with 37 C.F.R. § 1.104(d)(2)(e), call for the affidavit of the Examiner to set forth the facts within his knowledge which supposedly support this rejection.

**b) Camden I Does Not Claim the Same Patentable
Invention or an Obvious Variant of the Present
Invention**

Although not required to do so, Appellants nevertheless point out that the pending claims are not directed to the same patentable invention as the claims of Camden I or an obvious variant. In order for the Examiner's position to be proper, the claims of the present invention would need to be drawn to the same patentable invention as the claims of Camden I or an obvious variant.

Claim 12 of Camden I is represented below, in comparison with claims 13 and 60 of the present application, the independent claims. Points of commonality are shown with bold type, and underlining indicates points of distinction:

Claim 12 of Camden I and Claim 1 from Which it Depends	Claim 22 of the Present Invention	Claim 100 of the Present Invention
<p>1. A method of treating cancer selected from the group consisting of carcinoma, sarcoma, and lymphoma, the method comprising administering to a patient in need thereof a therapeutically effective amount of a composition comprising a benzimidazole compound of the formula:</p> <div data-bbox="224 766 544 940" data-label="Chemical-Block"> </div> <p>wherein X is hydrogen, halogen, alkyl of less than 7 carbon atoms, or alkoxy of less than 7 carbon atoms; n is a positive integer of less than 4; Y is hydrogen, chloro, nitro, methyl, ethyl, or oxychloro; R is hydrogen, an alkyl group of from 1 to 8 carbon atoms, or alkylaminocarbonyl wherein the alkyl group has from 3 to 6 carbon atoms; and R2 is NHCOOR1, wherein R1 is an aliphatic hydrocarbon of less than 7 carbon atoms; or a prodrug thereof, a pharmaceutically acceptable salt thereof, or mixtures thereof.</p> <p>12. A method of treating cancer according to claim 1, wherein the benzimidazole compound induces apoptosis in cancer cells at sub-lethal concentrations to normal cells.</p>	<p>22. A method for inducing apoptosis in a tumor cell expressing a tumor suppressor gene, comprising the steps of: (1) determining the tumor suppressor gene status of the tumor cell; and (2) administering an effective amount of a benzimidazole to said tumor cell, wherein expression of the tumor suppressor gene by the tumor cell and benzimidazole results in the apoptosis of the tumor cell.</p>	<p>100. A method for treating a patient having cancer, wherein cancer cells express a tumor suppressor, comprising the steps of: (1) determining the tumor suppressor gene status of the cancer cell; and (2) administering an effective amount of a benzimidazole to said patient, wherein the expression of the tumor suppressor gene by the cancer cell and the administration of the benzimidazole results in the inhibition of said cancer.</p>

As should immediately be evident, claim 1 of Camden I is distinct from present claim 100, which contains the following recitations:

1. cancer cells express a tumor suppressor
2. determining the tumor suppressor gene status of the cancer cell
3. the expression of the tumor suppressor gene by the cancer cell and the administration of the benzimidazole results in inhibition of said cancer.

Similarly, claims 1 and 12 of Camden I are distinct from present claim 22, which contains the following recitations:

4. expressing a tumor suppressor gene
5. expression of the tumor suppressor gene and [administration] of a benzimidazole result in apoptosis.

Thus, the claims cannot by definition be conflicting.

i. The Claims of Camden I Fail to Anticipate Appellants' Claims and Vice Versa

Independent claim 100 of the present invention recites a method of treating a patient having cancer where “*the cancer expresses a tumor suppressor*” (emphasis added). Moreover, claim 100 of the present invention requires “determining the *tumor suppressor gene status* of the cancer cell” (emphasis added). Finally, independent claim 100 of the present invention requires inhibition of cancer, and that this inhibition is the result of the expression of the tumor suppressor gene and the administration of a benzimidazole.

Furthermore, independent claim 22 of the present invention recites a method of inducing apoptosis in a tumor cell having cancer where “*the cancer expresses a tumor suppressor*” (emphasis added). Moreover, claim 22 of the present invention requires

“determining the *tumor suppressor gene status* of the cancer cell” (emphasis added). Finally, claim 22 of the present invention requires that the expression of the tumor suppressor gene and the benzimidazole result in apoptosis of the tumor cell.

In contrast, Camden I claim 1 only indicates that cancer in a patient can be treated with a composition comprising a particular benzimidazole. Likewise, Camden I claim 12, which depends from claim 1, only indicates that when cancer is treated in a patient by the administration of a particular benzimidazole, the compound induces apoptosis in cancer cells at sub-lethal concentrations to normal cells. However, neither claim 1 nor claim 12 of Camden I pertain to methods wherein *the cancer (or tumor cell) expresses a tumor suppressor*, or wherein the methods include *determining the tumor suppressor gene status of the tumor (or cancer) cell*. Finally, neither claim 1 nor claim 12 involve inhibition of cancer or induction of apoptosis by way of *the expression of the tumor suppressor gene by the tumor (or cancer) cell and the administration of a benzimidazole*. Therefore, the claims of Camden I fail to anticipate these features of the present invention for the simple reason that they do not recite any information pertaining to tumor suppressor gene status, methods of determining such status, or the combination of a benzimidazole and the tumor suppressor gene in the inhibition of cancer or in the induction of apoptosis.

Likewise, Appellants’ claims 22 and 100 do not recite a particular benzimidazole formula, nor do Appellants’ claims recite that such a benzimidazole formula induces apoptosis at sub-lethal concentrations to normal cells. Therefore, Appellants’ claims fail to anticipate the claims of Camden I.

**ii. The Claims of Camden I Fail to Render
Appellants' Claims Obvious and Vice Versa**

As discussed in the proceeding pages, there are limitations found in the claims of Camden I that are not found in the claims of the present invention. Additionally, there are numerous limitations found in the claims of the present invention that are not found in the claims of Camden I. Thus, novelty has been established. With regard to an analysis of patentable distinction, Appellants point out that to establish a *prima facie* case of obviousness, the claim must: (1) teach or suggest all the claim limitations; (2) provide some suggestion or motivation to modify the reference; and (3) provide a reasonable expectation of success. MPEP § 2142; *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

Presently, there is only one other reference cited – Perdomo – from which one could possibly derive the elements clearly missing from the Camden I claims. However, Perdomo cannot cure the deficiencies of the primary references (*e.g.*, wherein expression of the tumor suppressor gene and the administration of the benzimidazole result in inhibition of cancer or apoptosis of a tumor cell), as no such teaching in Perdomo can be found. Rather, Perdomo is merely being cited as teaching that determination of p53 status of cancer cells “could make it possible to predict the response to therapy [cisplatin] in certain patients.” July 10, 2005 Office Action, p. 3, para. 4 (Exhibit 6). Thus, Appellants’ claims cannot be deemed obvious over the claims of Camden I as there is simply no art of record to teach or suggest the claim limitations set forth above. Moreover, no discussion has been provided as to why one of skill in the art would equate the relationship between p53 and cisplatin with the limitations of the present invention

and therefore be motivated to combine the elements set forth in present claims 22 or 100. This factor constitutes an additional deficiency in the Examiner's analysis.

Furthermore, the Examiner has failed to even discuss the other required prong of the "two-way" test, namely, whether the claims of Camden I were rendered obvious by Appellants' claims. Indeed, Appellants' claims do not obviate the claims of Camden I as they fail to recite anything about the concentration at which to administer a benzimidazole. Thus, because Appellants' claims alone do not teach or suggest each limitation of the Camden I claims, there can be no finding of obviousness.

iii. Conclusion

In view of the foregoing, it is respectfully submitted that the Examiner erred in failing to consider the Third Declaration and address its impact on the rejections under 35 U.S.C. § 103(a).

Accordingly, because the Examiner has conceded that the Second Declaration of Tapas Mukhopadhyay, Sunil Chada, Abner Mhashilkar and Jack A. Roth Under 37 C.F.R. § 1.131 is sufficient to antedate the Camden I reference as to *in vitro* methods, and the Third Declaration of Tapas Mukhopadhyay, Sunil Chada, Abner Mhashilkar and Jack A. Roth Under 37 C.F.R. § 1.131 establishes reduction to practice of the *in vivo* methods, Camden I should not be considered prior art over the present application. Furthermore, the *claims* of Camden I and the *claims* of the present invention are patentably distinct. Under the current interference rules as well as controlling case law, the "two-way" patentability standard has not been met. The subject matter of claims 1 and 12 of Camden I *do not anticipate* the subject matter of claims 22 and/or 100 of the present application and *vice versa*. Therefore, Appellants respectfully request that the Board reverse the Examiner's rejections.

2. Perdomo Fails to Render the Claimed Inventions Obvious

In the absence of Camden I, Perdomo alone fails to render the claimed invention obvious because it does not teach or suggest each limitation of the claimed invention. In addition to the requirement that obviousness requires this showing, it also requires a showing that there was a motivation to combine the elements, and a likelihood of success in so doing. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

Perdomo does not teach or suggest the missing limitations that are not disclosed in Camden I. The Examiner has not shown where Perdomo includes any information pertaining to benzimidazoles or their effect on tumor cells. Further, Perdomo provides no motivation to one of ordinary skill in the art to provide the limitations since the reference pertains to cisplatin, a DNA damaging agent, *and not benzimidazoles*. As a result, the Examiner has not met his burden of establishing a *prima facie* case of obviousness.

Because Camden I is not available as prior art, and Perdomo fails to cure the deficiencies of the cited combination of references, the rejection of claims 76, 83-97 and 99-106 cannot be sustained. Therefore, Appellants respectfully request that the Board reverse the Examiner's rejections.

3. Even if Camden I Was Available as Prior Art, the Combination of Camden I in View of Perdomo Would Not Render the Claimed Invention Obvious

a) The Combination of Camden I in View of Perdomo Does Not Teach or Suggest All the Claim Limitations

A *prima facie* case of obviousness has not been established by the Examiner because the prior art references cited by the Examiner do not teach or suggest all of the claim limitations of the claims at issue in this rejection. In particular, the Examiner has not shown that Camden I teaches induction of apoptosis in a cell as the result of

expression of a tumor suppressor gene and the administration of a benzimidazole, or inhibition of cancer as the result of expression of a tumor suppressor gene and the administration of a benzimidazole. Furthermore, Perdomo does not teach or suggest the missing limitations that are not disclosed in Camden I. The Examiner has not shown where Perdomo pertains to or teaches benzimidazole administration. Instead, it appears that Perdomo pertains to inhibition or apoptosis of cancer cells expressing wild-type p53 through the use of cisplatin and/or radiation.

b) The Combination of Camden I in View of Perdomo Provides No Motivation to One of Ordinary Skill in the Art to Provide for the Claimed Invention

Camden I in view of Perdomo does not provide a person of ordinary skill in the art a motivation to combine. Perdomo states that “treatment with cisplatin and radiation did not reduce the size of mt-p53 tumors, while wt-p53 tumors regressed by approximately 60%.” (abstract). Conversely, Camden I is said to teach selectivity in killing p53 abnormal cell lines versus cells expressing normal p53. Furthermore, in a comment regarding the ability of carbendazim to kill cancer cells, Camden I states “Carbendazim is equally effective against p53 deficient/defective cell lines, unlike most existing drugs.” (col. 11, lines 47-50). Therefore, one of ordinary skill in the art would *not* have been motivated to use the benzimidazole derivatives taught by Camden I to induce apoptosis or inhibit cancer after confirming that the cells *are expressing* the p53 gene. Rather, it would seem that the combination of these references actually *teaches away* from the present invention.

Accordingly, because the Examiner has not met his burden of proof in establishing a *prima facie* case of obviousness of claims over the combination of Camden

I in view of Perdomo, Appellants respectfully request that the Board reverse the Examiner's rejections.

C. Claims 76-77, 83-97 and 99-106 Are Not Rendered Obvious Over Camden I in View of Perdomo in Further View of Delatour Under 35 U.S.C. § 103(a)

In the July 10, 2006 Office Action, the Examiner rejected claims 76-77, 83-97 and 99-106 as obvious under 35 U.S.C. § 103(a) over Camden I in view of Perdomo in further view of Delatour (Exhibit 7).

As described above, the discussion of which is herein incorporated into this section, Camden I is not available as prior art. Regardless, the Examiner has not met his burden of proof in establishing a *prima facie* case of obviousness because he has not established that the combination of Camden I, Perdomo and Delatour teaches or suggests each limitation of the claimed invention.

Also as discussed above, Camden I and Perdomo do not teach or suggest each limitation of the claimed invention. Additionally, there is no motivation to combine the references of Camden I and Perdomo, as such a combination would teach away from the claimed invention. The inclusion of Delatour fails to cure this deficiency. Delatour does not include any information pertaining to the tumor suppressor genes or determining their status prior to the administration of a benzimidazole. Additionally, Delatour does not teach or suggest inhibition of cancer as a result of expression of a tumor suppressor and the administration of a benzimidazole. Therefore, Appellants respectfully request that the Board reverse the Examiner's rejection 76-77, 83-97 and 99-106 as obvious under 35 U.S.C. § 103(a) over Camden I in view of Perdomo in further view of Delatour.

D. Claims 2, 10, 15-19, 21-29, 76, 83, 85, 88-97 and 100-106 Are Not Rendered Obvious Over Camden II as Evidenced by Camden I in View of Perdomo Under 35 U.S.C. § 103(a)

The Examiner has failed to establish a *prima facie* case of obviousness based on Camden II (Exhibit 8) as evidenced by Camden I in view of Perdomo. In the July 10, 2006 Office Action, the Examiner rejects claims 2, 10, 15-19, 21-29, 76, 83, 85, 88-97 and 100-106 under 35 U.S.C. § 103 on these grounds. According to the Examiner, it would be obvious to determine the status of a tumor suppressor gene in a tumor cell prior to administering a benzimidazole derivative using analytical methods because Camden I “teaches the selectivity in killing p53 abnormal cell lines versus cells expressing normal p53 (col. 12, lines 52+), while Perdomo [*sic*] *et al.* teaches that the ‘response to cisplatin *in vivo* of tumors derived from different NSCLC lines was dependent on p53 status (p. 17, col. 1, para. 2).” July 10, 2006 Office Action, pp 7-8 (Exhibit 6).

Appellants note that Camden I is unavailable as prior art. Regardless, the Examiner has failed to establish a *prima facie* case of obviousness based on Camden II as evidenced by Camden I in view of Perdomo.

1. The Combination of Camden II as Evidenced by Camden I in View of Perdomo Does Not Teach or Suggest All the Claim Limitations

As discussed above, the discussion of which is incorporated into this section, the combination of Camden I and Perdomo does not teach or suggest each limitation of the claimed invention. Camden II does not teach that the administration of benzimidazole induces apoptosis; Camden II does not characterize the tumor cell lines as expressing a tumor suppressor gene such as p53. The Examiner has not shown where Camden II even mentions tumor suppressor genes. Additionally, the Examiner has not shown that Camden I or II teaches induction of apoptosis in a cell as the result of expression of a

tumor suppressor gene and the administration of a benzimidazole, or inhibition of cancer as the result of expression of a tumor suppressor gene and the administration of a benzimidazole. Perdomo does not teach or suggest the missing limitations that are not disclosed in Camden I or Camden II. The Examiner has not shown where Perdomo pertains to or teaches benzimidazole administration. The addition of Camden II fails to cure this deficiency. Appellants have found no disclosure in Camden II even pertaining to tumor suppressor genes.

2. The Combination of Camden II as Evidenced by Camden I in View of Perdomo Provides No Motivation to One of Ordinary Skill in the Art to Provide for the Claimed Invention

Camden II as evidenced by Camden I in view of Perdomo does not provide a person of ordinary skill in the art a motivation to combine. Perdomo states that “treatment with cisplatin and radiation did not reduce the size of mt-p53 tumors, while wt-p53 tumors regressed by approximately 60%.” (abstract). Conversely, Camden I is said to teach selectivity in killing p53 abnormal cell lines versus cells expressing normal p53. Furthermore, in a comment regarding the ability of carbendazim to kill cancer cells, Camden I states “Carbendazim is equally effective against p53 deficient/defective cell lines, unlike most existing drugs.” (col. 11, lines 47-50). Therefore, one of ordinary skill in the art would *not* have been motivated to use the benzimidazole derivatives taught by Camden I to induce apoptosis or inhibit cancer after confirming that the cells *are expressing* the p53 gene. It would seem that the combination of these references actually *teaches away* from the present invention. The addition of Camden II adds nothing to motivate a person of ordinary skill in the art as the reference appears silent regarding tumor suppressor genes.

Therefore, in view of the fact that the Examiner has failed to establish a *prima facie* case of obviousness over Camden II as evidenced by Camden I in view of Perdomo, Appellants respectfully request that the Board reverse the Examiner's rejections of claims 2, 10, 15-19, 21-29, 76, 83, 85, 88-97 and 100-106 on these grounds.

E. Claims 3 and 77 Are Not Rendered Obvious Over Camden II in View of Perdomo in Further View of Either Delatour or Nasr Under 35 U.S.C. § 103(a)

The Examiner has not met his burden of proof in establishing a *prima facie* case of obviousness of claims over Camden II in view of Perdomo and in further view of either Delatour or Nasr (Exhibit 9). In the July 10, 2006 Office Action, the Examiner rejected claims 3 and 77 on these grounds.

Alone, the combination of Camden II and Perdomo does not provide a person of ordinary skill in the art a motivation to combine the references. Alone, these two references fail to render the claimed invention obvious because they do not teach or suggest each limitation of the claimed invention nor do they provide any suggestion or motivation to combine reference teachings. The addition of Delatour or Nasr fails to cure this deficiency.

1. The Combination of Camden II in View of Perdomo in Further View of Either Delatour or Nasr Does Not Teach or Suggest All the Claim Limitations

As discussed in the previous section, the discussion of which is incorporated into this section, the Examiner has not shown and Appellants cannot find any mention of tumor suppressor genes in Camden II. The Examiner has not shown nor can Appellants find any mention in Camden II pertaining to the administration of a benzimidazole wherein benzimidazole administration and the expression of a tumor suppressor result in

apoptosis of a tumor or inhibition of cancer. Perdomo does not appear to pertain to administration of benzimidazoles at all.

Neither Delatour nor Nasr cure the deficiencies of Camden II and Perdomo. The Examiner has not presented any discussion in Delatour or Nasr pertaining to tumor suppressors or pertaining to the administration of a benzimidazole wherein benzimidazole administration and the expression of a tumor suppressor result in apoptosis of a tumor or inhibition of cancer.

2. The Combination of Camden II in View of Perdomo in Further View of Either Delatour or Nasr Provides No Motivation to One of Ordinary Skill in the Art to Provide for the Claimed Invention

The Examiner, in addition to failing to meet his burden of proof in establishing a *prima facie* case of obviousness with respect to the combination of Camden II in view of Perdomo and further in view of Delatour or Nasr teaching or suggesting each claim limitation, the Examiner has failed to establish that a person of ordinary skill would be motivated to combine the references to practice the claimed invention.

As discussed above, the Examiner has not shown where Camden II, Delatour or Nasr, even mention tumor suppressors. Perdomo does not provide the missing motivation to combine the references. The Examiner has previously indicated that Perdomo teaches “the response to cisplatin *in vivo* of tumors derived from different NSCLC lines was dependent on p53 status[.]” July 10, 2006 Office Action, p. 4, para. 2 (Exhibit 6). However, it appears that Perdomo only discloses the use of DNA damaging agents such as cisplatin and radiation. “Cisplatin, a DNA-cross-linking chemotherapeutic drug, and radiation, a treatment that produces single-strand breaks in DNA, are widely used to treat locally advanced non-small-cell lung cancer (NSCLC).” Perdomo, p. 11,

col. 1, para. 2 (Exhibit 2). In contrast, the present application states that “[i]t is currently believed that BZs exert their cytotoxic effects by binding to the microtubule system and disrupting its functions[.]” Specification, p. 10, lines 1-3. The Examiner has not indicated why one would be motivated to check the status of tumor suppressor genes in a tumor prior to the administration of a microtubule disruptor in the same manner as one might check the status of tumor suppressor genes prior to the administration of a DNA damaging agent. Accordingly, the Examiner has not met his burden of proof in establishing a *prima facie* case of obviousness, as he has not shown any suggestion or motivation to combine reference teachings.

Therefore, it is respectfully requested that the Board reverse the Examiner’s rejection of claims 3 and 77 under 35 U.S.C. § 103 over Camden II in view of Perdomo in further view of either Delatour or Nasr.

F. Claims 13-14 and 86-87 Are Not Rendered Obvious Over Camden II in View of Perdomo and Further in View of Lucci Under 35 U.S.C. § 103(a)

The Examiner has not met his burden of proof in establishing a *prima facie* case of obviousness of claims over Camden II in view of Perdomo and further in view of Lucci (Exhibit 10) under 35 U.S.C. § 103. In the July 10, 2006 Office Action, the Examiner rejected claims 13-14 and 86-87 on these grounds.

As discussed above, the discussion of which is incorporated into this section, the Examiner has failed to establish a *prima facie* case of obviousness based on Camden II and Perdomo. Lucci does nothing to cure this deficiency. In order to establish a *prima facie* case of obviousness, the references must teach or suggest each claim limitation. In addition to the requirement that obviousness requires this showing, it also requires a

showing that there was a motivation to combine the elements, and a likelihood of success in so doing. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

As discussed above, the combination of Camden II and Perdomo fails to teach or suggest each limitation of the claimed invention. Additionally, the combination of Camden II and Perdomo does not provide a person of ordinary skill in the art the requisite motivation to combine the references. Lucci fails to cure either the deficiency of teaching or suggesting each limitation or the deficiency of lack of motivation.

Regarding teaching or suggesting each limitation of the claimed invention, the Examiner has not shown, that the combination of Camden II, Perdomo and Lucci teaches or suggests determining the tumor suppressor status of a cancer cell prior to the administration of a benzimidazole, or inhibition of cancer or induction of apoptosis as a result of the administration of a benzimidazole and the expression of a tumor suppressor. Appellants do identify any mention in Lucci pertaining to either benzimidazoles or tumor suppressor gene status. In fact, it appears that the only non-conventional chemotherapy drugs disclosed in Lucci were drugs which bind to P-glycoprotein, a plasma membrane drug efflux pump.

The greatly reduced sensitivity to anticancer drugs, a hallmark of MDR, often results from overexpression of P-glycoprotein (P-gp), a 170-kilodalton (kDa) plasma membrane protein that functions as a drug efflux pump....A major challenge in cancer chemotherapy is to delineate the molecular mechanisms by which MDR modulators, *e.g.* tamoxifen, cyclosporine A, and SDZ PSC 833 (PSC 833)...reverse drug resistance. These agents have been shown to bind directly to P-gp and thereby interfere with binding and export of anticancer drugs.

Lucci *et al.*, p. 301, left col., para. 2 (Exhibit 10).

Lucci additionally fails to cure the deficiency of Camden II and Perdomo as it relates to a lack of motivation to combine. Lucci discloses non-conventional

chemotherapeutic agents *which are not benzimidazoles*. The Examiner has not shown how the administration of the non-conventional chemotherapeutic agents as taught by Lucci would provide a person of ordinary skill in the art the requisite motivation to practice the claimed invention given the deficiencies of the combination of Camden II and Perdomo.

Thus, Camden II in view of Perdomo and further in view of Lucci provides no motivation to combine the cited references. Therefore, Appellants respectfully request that the Board reverse the Examiner's rejections of claims 13-14 and 86-87 under 35 U.S.C. § 103.

CONCLUSION

For the above-argued reasons, Appellants respectfully request that the Board reverse the Examiner's rejections of the claims. Appellants have provided arguments that overcome the pending rejections. Appellants respectfully submit that the Office Action's conclusion that the claims should be rejected is unwarranted. It is therefore again requested that the Board overturn the Action's rejections.

Respectfully submitted,



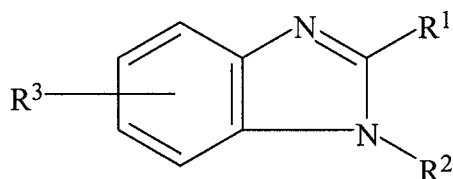
FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas 78701
512.474.5201 (telephone)
512.536.4598 (fax)
Date: December 8, 2006

Tamara A. Kale
Reg. No. 53,087
Attorney for Applicants

VIII. CLAIMS APPENDIX

1. (Canceled)

2. (Previously Presented) The method of claim 22, wherein the benzimidazole is a derivative having the formula:



wherein R³ is selected from the group consisting of H, carboxyl (-CO₂H), hydroxyl, amino, chloro, difluoromethoxy, benzoyl, phenyl-thio, pyridinyl, propyl-thio, diphenyl, methoxy, fluorophenylmethyl-2-chloro, propenyl, chloropropyl or esters (-CO₂R⁴) wherein R⁴ is selected from the group consisting of alkoxy, haloalkyl, alkenyl, and cycloalkyl, wherein the alkyl groups have from 1 – 8 carbons, or CH₃CH₂(OCH₂CH₂)_n—, or CH₃CH₂CH₂(OCH₂CH₂CH₂)_n—, or (CH₃)₂CH(OCH(CH₃)CH₂)_n—, wherein n is from 1 – 3, wherein R¹ is OH, Cl, SH, (methoxy-dimethyl pyridinyl)methyl-(sulfonyl), carbamate or piperidin-4-yl, and R² is hydrogen, α-methylvinyl, 3-chloropropyl or piperidin-4-yl, or the pharmaceutically effective organic or inorganic salts thereof, or mixtures thereof.

3. (Original) The method of claim 2, wherein the benzimidazole derivative is methyl 5-benzoylbenzimidazole-2-carbamate (mebendazole).

4-8. (Canceled)

9. (Previously Presented) The method of claim 22, wherein the dose of benzimidazole is at least 0.05 mg per kg body weight.

10. (Previously Presented) The method of claim 22, wherein benzimidazole administration is repeated at least once.

11-12. (Canceled)

13. (Previously Presented) The method of claim claim 22, wherein the tumor cell is a multidrug resistant tumor cell.

14. (Original) The method of claim 13, wherein the multidrug resistant tumor cell is a lung tumor cell, a non-small cell lung carcinoma cell, a breast cancer cell, or a sarcoma cell.

15. (Previously Presented) The method of claim 22, wherein the tumor cell is a lung tumor cell.

16. (Original) The method of claim 15, wherein the lung tumor cell is a non-small cell lung carcinoma cell.

17. (Previously Presented) The method of claim 22, wherein the tumor cell is a breast cancer cell.

18. (Previously Presented) The method of claim 22, wherein the tumor cell is a sarcoma cell.

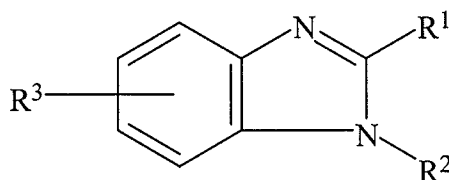
19. (Previously Presented) The method of claim 22, wherein the tumor suppressor gene is *p53*, *p16*, *p21*, *Rb*, *p15*, *BRCA1*, *BRCA2*, *zac1*, *p73*, *ATM*, *HIC-1*, *DPC-4*, *FHIT*, *NF2*, *APC*, *DCC*, *PTEN*, *ING1*, *NOEY1*, *NOEY2*, *PML*, *OVCA1*, *MADR2*, *WT1*, *53BP2*, *IRF-1*, *MDA-7* and *C-CAM*.

20. (Previously Presented) The method of claim 22, wherein the tumor suppressor gene is MDA-7.
21. (Previously Presented) The method of claim 22, wherein the tumor suppressor gene is *p53*.
22. (Previously Presented) A method for inducing apoptosis in a tumor cell expressing a tumor suppressor gene, comprising the steps of:
- (1) determining the tumor suppressor gene status of the tumor cell; and
 - (2) administering an effective amount of a benzimidazole to said tumor cell,
- wherein expression of the tumor suppressor gene by the tumor cell and benzimidazole results in the apoptosis of the tumor cell.
23. (Original) The method of claim 22, wherein determining comprises Southern blotting.
24. (Original) The method of claim 22, wherein determining comprises Northern blotting.
25. (Original) The method of claim 22, wherein determining comprises PCR.
26. (Original) The method of claim 22, wherein determining comprises ELISA.
27. (Original) The method of claim 22, wherein determining comprises Western blotting.
28. (Original) The method of claim 22, wherein determining comprises immunofluorescence.

29. (Previously Presented) The method of claim 22, wherein the tumor cell expresses a functional tumor suppressor gene.

30-75.

76. (Previously Presented) The method of claim 100, wherein the benzimidazole is a derivative having the formula:



wherein R³ is selected from the group consisting of H, carboxyl (-CO₂H), hydroxyl, amino, chloro, difluoromethoxy, benzoyl, phenyl-thio, pyridinyl, propyl-thio, diphenyl, methoxy, fluorophenylmethyl-2-chloro, propenyl, chloropropyl or esters (-CO₂R⁴) wherein R⁴ is selected from the group consisting of alkoxy, haloalkyl, alkenyl, and cycloalkyl, wherein the alkyl groups have from 1 – 8 carbons, or CH₃CH₂(OCH₂CH₂)_n—, or CH₃CH₂CH₂(OCH₂CH₂CH₂)_n—, or (CH₃)₂CH(OCH(CH₃)CH₂)_n—, wherein n is from 1 – 3, wherein R¹ is OH, Cl, SH, (methoxy-dimethyl,pyridinyl)methyl-(sulfonyl), carbamate or piperidin-4-yl, and R² is hydrogen, α-methylvinyl, 3-chloropropyl or piperidin-4-yl, or the pharmaceutically effective organic or inorganic salts thereof, or mixtures thereof.

77. (Previously Presented) The method of claim 100, wherein the benzimidazole derivative is methyl 5-benzoylbenzimidazole-2-carbamate (mebendazole).

78-82.

83. (Previously Presented) The method of claim 100, wherein the tumor suppressor gene is p53, p16, p21, Rb, p15, BRCA1, BRCA2, zac1, p73, ATM, HIC-1, DPC-4, FHIT, NF2, APC, DCC, PTEN, ING1, NOEY1, NOEY2, PML, OVCA1, MADR2, WT1, 53BP2, IRF-1, MDA-7 and C-CAM.

84. (Previously Presented) The method of claim 100, wherein the tumor suppressor gene is MDA-7.

85. (Previously Presented) The method of claim 100, wherein the tumor suppressor gene is *p53*.

86. (Previously Presented) The method of claim 100, wherein the cancer cell is a multidrug resistant tumor cell.

87. (Original) The method of claim 86, wherein the multidrug resistant tumor cell is a lung tumor cell, a non-small cell lung carcinoma cell, a breast cancer cell, or a sarcoma cell.

88. (Previously Presented) The method of claim 100, wherein the cancer cell is a lung tumor cell.

89. (Original) The method of claim 88, wherein the lung tumor cell is a non-small cell lung carcinoma cell.

90. (Previously Presented) The method of claim 100, wherein the cancer cell is a breast cancer cell.

91. (Previously Presented) The method of claim 100, wherein the cancer cell is a sarcoma cell.

92. (Previously Presented) The method of claim 100, wherein benzimidazole administration comprises intratumoral administration.

93. (Previously Presented) The method of claim 100, wherein benzimidazole administration comprises systemic administration.

94. (Previously Presented) The method of claim 100, wherein benzimidazole administration comprises oral administration.

95. (Previously Presented) The method of claim 100, wherein benzimidazole administration comprises administration in the area local to a tumor in said patient.

96. (Previously Presented) The method of claim 100, wherein benzimidazole administration comprises administration in the area regional to a tumor in said patient.

97. (Previously Presented) The method of claim 100, wherein benzimidazole administration is repeated at least once.

98. (Previously Presented) The method of claim 100, wherein the dose of benzimidazole is about 0.1 mg per kg body weight.

99. (Previously Presented) The method of claim 100, wherein the dose of benzimidazole is about 1.0 mg per kg body weight.

100. (Previously Presented) A method for treating a patient having cancer, wherein cancer cells express a tumor suppressor, comprising the steps of:

(1) determining the tumor suppressor gene status of the cancer cell; and

(b) administering an effective amount of a benzimidazole to said patient, wherein the expression of the tumor suppressor gene by the cancer cell and the administration of the benzimidazole results in the inhibition of said cancer.

101. (Original) The method of claim 100, wherein determining comprises Southern blotting.

102. (Original) The method of claim 100, wherein determining comprises Northern blotting.

103. (Original) The method of claim 100, wherein determining comprises PCR.
104. (Original) The method of claim 100, wherein determining comprises ELISA.
105. (Original) The method of claim 100, wherein determining comprises Western blotting.
106. (Original) The method of claim 100, wherein determining comprises immunofluorescence.
- 107-184. (Canceled)

IX. EVIDENCE APPENDIX

1. Camden (U.S. Patent 6,262,093, "Camden I"); first cited in March 18, 2005 Office Action.
2. Perdomo *et al.*, *J. Cancer Res. Clin. Oncol.*, 124:10-18, 1998; first cited in March 18, 2005 Office Action.
3. Second Declaration of Tapas Mukhopadhyay, Sunil Chada, Abner Mhashilkar and Jack A. Roth under 37 C.F.R. § 1.131; mailed August 18, 2005.
4. Office Action dated November 16, 2005.
5. Third Declaration of Tapas Mukhopadhyay, Sunil Chada, Abner Mhashilkar and Jack A. Roth under 37 C.F.R. § 1.131; mailed April 17, 2006.
6. Office Action dated July 10, 2006.
7. Delatour *et al.*, *Therapie*, 31:505-515, 1976; first cited in March 18, 2005 Office Action.
8. Camden (U.S. Patent 5,880,144, "Camden II"); first cited in July 10, 2006 Office Action.
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10. Lucci *et al.*, *Cancer*, 86:300-311, 2000; first cited in July 10, 2006 Office Action.

X. RELATED PROCEEDINGS APPENDIX

[NONE]

EXHIBIT 1



US006262093B1

(12) United States Patent
Camden**(10) Patent No.: US 6,262,093 B1**
(45) Date of Patent: *Jul. 17, 2001**(54) METHODS OF TREATING CANCER WITH**
BENZIMIDAZOLES**(75) Inventor: James Berger Camden, West Chester,**
OH (US)**(73) Assignee: The Proctor & Gamble Company,**
Cincinnati, OH (US)**(*) Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: 09/264,942**(22) Filed: Mar. 9, 1999****Related U.S. Application Data****(63)** Continuation-in-part of application No. 08/927,550, filed on Sep. 6, 1997, now Pat. No. 5,880,144, and a continuation-in-part of application No. 09/081,384, filed on May 19, 1998, now abandoned, and a continuation-in-part of application No. 09/081,627, filed on May 19, 1998, now abandoned, said application No. 08/927,550, is a division of application No. 08/771,193, filed on Dec. 20, 1996, now Pat. No. 5,767,138, which is a division of application No. 08/420,914, filed on Apr. 12, 1995, now abandoned.**(51) Int. Cl.⁷ A61K 31/425; A61K 31/415****(52) U.S. Cl. 514/365; 514/396; 514/397;**
514/398; 514/399; 514/400; 514/388; 424/450**(58) Field of Search 514/365, 388,**
514/396, 397, 398, 399, 400; 424/450**(56) References Cited****U.S. PATENT DOCUMENTS**3,010,968 11/1961 Loux 260/309.2
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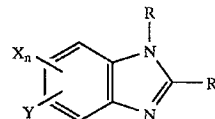
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Primary Examiner—Frederick Krass**(74) Attorney, Agent, or Firm—Rose Ann Dabek; Steven W. Miller****(57) ABSTRACT**

This invention is a method of treating cancer, both carcinomas and sarcomas, and viral infections, in particular HIV through the administration of a pharmaceutical composition containing a benzimidazole derivative. The composition is also claimed. The benzimidazole derivative is selected from the group consisting of:

wherein X_n is hydrogen, halogen, alkyl of less than 7 carbon atoms or alkoxy of less than 7 carbon atoms; n is a positive integer of less than 4; Y is hydrogen, chlorine, nitro, methyl, ethyl or oxychloro; and R is hydrogen, alkylaminocarbonyl wherein the alkyl group has from 3 to 6 carbon atoms, or an alkyl group of from 1 to 8 carbon atoms and R_2 is 4-thiazolyl, NHCOOR , wherein R_1 is aliphatic hydrocarbon of less than 7 carbon atoms, prodrugs, pharmaceutically acceptable salts and mixtures thereof and a pharmaceutically acceptable carrier**36 Claims, No Drawings**

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METHODS OF TREATING CANCER WITH BENZIMIDAZOLES

This application is a continuation-in-part of application Ser. No. 08/927,550, filed Sep. 6, 1997, now U.S. Pat. No. 5,880,144, which is a divisional of application Ser. No. 08/771,193, filed Dec. 20, 1996, now U.S. Pat. No. 5,767,138, which is a divisional of application Ser. No. 08/420,914, filed Apr. 12, 1995, now abandoned. This application is also a continuation-in-part of application Ser. No. 09/081,384, filed May 19, 1998, now abandoned, and also a continuation-in-part of application Ser. No. 09/081,627, filed May 19, 1998, now abandoned.

TECHNICAL FIELD

This invention is a method of treating cancer, both carcinomas and sarcomas, and viral infections, in particular HIV through the administration of a pharmaceutical composition containing a benzimidazole derivative. The composition is also claimed.

BACKGROUND OF THE INVENTION

Cancers are the leading cause of death in animals and humans. The exact cause of cancer is not known, but links between certain activities such as smoking or exposure to carcinogens and the incidence of certain types of cancers and tumors has been shown by a number of researchers.

Many types of chemotherapeutic agents have been shown to be effective against cancers and tumor cells, but not all types of cancers and tumors respond to these agents. Unfortunately, many of these agents also destroy normal cells. The exact mechanism for the action of these chemotherapeutic agents are not always known.

Despite advances in the field of cancer treatment the leading therapies to date are surgery, radiation and chemotherapy. Chemotherapeutic approaches are said to fight cancers that are metastasized or ones that are particularly aggressive. Such cytotoxic or cytostatic agents work best on cancers with large growth factors, i.e., ones whose cells are rapidly dividing. To date, hormones, in particular estrogen, progesterone and testosterone, and some antibiotics produced by a variety of microbes, alkylating agents, and anti-metabolites form the bulk of therapies available to oncologists. Ideally cytotoxic agents that have specificity for cancer and tumor cells while not affecting normal cells would be extremely desirable. Unfortunately, none have been found and instead agents which target especially rapidly dividing cells (both tumor and normal) have been used.

The development of materials that would target tumor cells due to some unique specificity for them would be a breakthrough. Alternatively, materials that were cytotoxic to tumor cells while exerting mild effects on normal cells would be desirable.

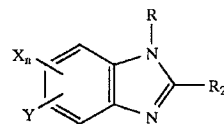
HIV and other viral infections are another leading cause of death. HIV is a disease in which a virus is replicated in the body which attacks the body's immune system. The HIV virus is not easily destroyed nor is there a good mechanism for keeping the host cells from replicating the virus. Herpes Simplex is another viral infection which is difficult, if not impossible, to cure. A method of treating these diseases and other viral infections is highly desirable. A material which would target the HIV virus and inhibit viral replication is highly desirable.

The benzimidazole derivatives used herein to treat cancer and/or viral infection have been used as fungicides and as anthelmintics.

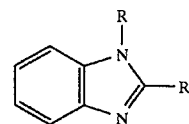
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SUMMARY OF THE INVENTION

A method of treating cancer, in particular, treating cancers in warm blooded animals and humans, comprising administering a therapeutically effective amount of a composition comprising a benzimidazole compound selected from the group consisting of:



wherein X is hydrogen, halogen, alkyl of less than 7 carbon atoms or alkoxy of less than 7 carbon atoms; n is a positive integer of less than 4; Y is hydrogen, chlorine, nitro, methyl, ethyl or oxychloro; R is hydrogen, alkylaminocarbonyl wherein the alkyl group has from 3 to 6 carbon atoms, or an alkyl group of from 1 to 8 carbon atoms and R₂ is 4-thiazolyl, NHCOOR₁ wherein R₁ is aliphatic hydrocarbon of less than 7 carbon atoms, and preferably an alkyl group of less than 7 carbon atoms is claimed. Preferably the compositions contain:



wherein R is an alkyl of 1 through 8 carbon atoms and R₂ is selected from the group consisting of 4-thiazolyl, NHCOOR₁, wherein R₁ is methyl, ethyl or isopropyl and the non-toxic, pharmaceutically acceptable acid salts with both organic and inorganic acids. The most preferred compounds are 2-(4-thiazolyl)benzimidazole, methyl-(butylcarbamoyl)-2-benzimidazolecarbamate and 2-methoxycarbonylamino-benzimidazole and those wherein Y is chloro.

In the present invention it has been discovered that the compounds described above are useful for the inhibition of HIV and the treatment of HIV infection and similar retrovirus infections. The present invention also provides methods for the treatment of HIV infection comprising administering to a host infected with HIV a pharmaceutically or therapeutically effective or acceptable amount of a compound as described above, particularly those wherein R is 4-thiazolyl.

The present invention also provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a compound as described above.

These compositions have been discovered to inhibit the growth of cancer or other tumors in humans or animals and to induce apoptosis of cancer cells by administration of a therapeutically effective amount of the composition, preferably by administering a benzimidazole compound to the site of the cancer.

More specifically, this invention provides an anti-cancer composition comprising a pharmaceutical carrier and a benzimidazole derivative as defined herein along with a method for treating such cancers. These compositions can induce apoptosis in cancer cells.

These compositions are also effective against viruses and are used to treat viral infections and this invention provides a method of treating viral infections such as herpes, hepatitis, influenza and rhinoviruses.

DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

As used herein, a "pharmaceutically acceptable" component is one that is suitable for use with humans and/or animals without undue adverse side effects (such as toxicity, irritation, and allergic response) commensurate with a reasonable benefit/risk ratio.

As used herein, the term "safe and effective amount" refers to the quantity of a component which is sufficient to yield a desired therapeutic response without undue adverse side effects (such as toxicity, irritation, or allergic response) commensurate with a reasonable benefit/risk ratio when used in the manner of this invention. By "therapeutically effective amount" is meant an amount of a compound of the present invention effective to yield the desired therapeutic response. For example to inhibit HIV infection or treat the symptoms of infection in a host or an amount effective to delay the growth of or to cause a cancer, either a sarcoma or lymphoma, to shrink. The specific safe and effective amount or therapeutically effective amount will, vary with such factors as the particular condition being treated, the physical condition of the patient, the type of mammal being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the compounds or its derivatives.

As used herein, a "pharmaceutical salts" is salt of the benzimidazole derivatives which are modified by making acid or base salts of the compounds. Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids. Preferably the salts are made using an organic or inorganic acid. These preferred acid salts are chlorides, bromides, sulfates, nitrates, phosphates, sulfonates, formates, tartrates, maleates, malates, citrates, benzoates, salicylates, ascorbates, and the like.

As used herein, a "pharmaceutical carrier" is a pharmaceutically acceptable solvent, suspending agent or vehicle for delivering the benzimidazole derivatives to the animal or human. The carrier may be liquid or solid and is selected with the planned manner of administration in mind.

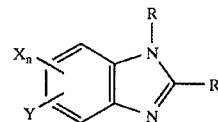
As used herein, "cancer" refers to all types of cancer or neoplasm or malignant tumors found in mammals, including carcinomas and sarcomas. Examples of cancer are cancer of the brain, breast, cervix, colon, head & neck, kidney, lung, non-small cell lung, melanoma, mesothelioma, ovary, sarcoma, stomach, uterus and Medulloblastoma.

As used herein, the "benzimidazole derivatives" are the benzimidazoles, and their salts and also their prodrugs. The exact benzimidazoles are described in detail below. The preferred materials are the products sold under the names "Thiabendazole®", "Benomyl®" and "Carbendazim®" by BASF and Hoechst, DuPont and MSD-AgVet.

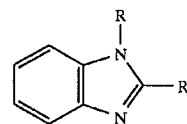
As used herein "viruses" includes viruses which infect animals or mammals, including humans. Viruses includes retroviruses, HIV, influenza, polio viruses, herpes, herpes simplex, rhinoviruses, hepatitis, and the like.

B. The Benzimidazole Derivatives

The benzimidazole derivatives which are known for their antifungal activities. They are systemic fungicides used to prevent and eradicate fungi. The compounds have the following structure:



wherein X is hydrogen, halogen, alkyl of less than 7 carbon atoms or alkoxy of less than 7 carbon atoms; n is a positive integer of less than 4; Y is hydrogen, chlorine, nitro, methyl or ethyl; R is hydrogen, alkylaminocarbonyl wherein the alkyl group has from 3 to 6 carbon atoms or an alkyl group having from 1 to 8 carbons, and R₂ is 4-thiazolyl, NHCOOR₁ wherein R₁ is aliphatic hydrocarbon of less than 7 carbon atoms, and preferably and alkyl group of less than 7 carbon atoms. Preferably the compositions are:



wherein R is an alkyl of 1 through 8 carbon atoms and R₂ is selected from the group consisting of 4-thiazolyl, NHCOOR₁, wherein R₁ is methyl, ethyl or isopropyl and the non-toxic, pharmaceutically acceptable acid salts with both organic and inorganic acids.

The most preferred compounds are 2-(4-thiazolyl) benzimidazole, methyl-(butylcarbamoyl)-2-benzimidazolecarbamate and 2-methoxycarbonylamino-benzimidazole and the compounds wherein Y is chloro and X is hydrogen.

The benzimidazole compounds also include prodrugs. "Prodrugs" are considered to be any covalently bonded carriers which release the active parent drug according to the formula of the benzimidazole derivatives described above in vivo when such prodrug is administered to a mammalian subject. Prodrugs of the benzimidazole compounds are prepared by modifying functional groups present in the compounds in such a way that the modifications are cleaved, either in routine manipulation or in vivo, to the parent compounds. Prodrugs include compounds wherein hydroxy, amino, or sulfhydryl groups are bonded to any group that, when administered to a mammalian subject, cleaves to form a free hydroxyl, amino, or sulfhydryl group, respectively. Examples of prodrugs include, but are not limited to, acetate, formate, or benzoate derivatives of alcohol and amine functional groups in the benzimidazole derivatives; phosphate esters, dimethylglycine esters, aminoalkylbenzyl esters, aminoalkyl esters and carboxyalkyl esters of alcohol and phenol functional groups in the benzimidazole derivatives; and the like.

The pharmaceutically acceptable salts of the benzimidazole derivatives include the conventional non-toxic salts or the quaternary ammonium salts of the benzimidazole derivatives formed, for example, from non-toxic inorganic or organic acids. For example, such conventional non-toxic salts include those derived from inorganic acids such as hydrochloric, hydrobromic, sulfuric, sulfamic, phosphoric, nitric and the like; and the salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, pantoic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicylic, sulfanilic, 2-acetoxybenzoic, fumaric, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isethionic, and the like.

The pharmaceutically acceptable salts of the present invention are synthesized from the benzimidazole derivatives which contain a basic or acidic moiety by conventional chemical methods. Generally, such salts are prepared by reacting the free acid or base forms of these compounds with a stoichiometric amount of the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, nonaqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred. Lists of suitable salts are found in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, Pa., 1985, p. 1418, the disclosure of which is hereby incorporated by reference. The disclosures of all of the references cited herein are hereby incorporated herein by reference in their entirety.

Synthesis

The benzimidazole derivatives are prepared in a number of ways well known to one skilled in the art of organic synthesis. The benzimidazole derivatives are synthesized using the methods described below, together with synthetic methods known in the art of synthetic organic chemistry, or variations thereon as appreciated by those skilled in the art. Preferred methods include but are not limited to those methods described below. Each of the references cited below are hereby incorporated herein by reference.

These compounds are prepared according to the method described in U.S. Pat. No. 3,738,995 issued to Adams et al, Jun. 12, 1973. The thiazolyl derivatives are prepared according to the method described in Brown et al., *J. Am. Chem. Soc.*, 83, 1764 (1961) and Grenda et al., *J. Org. Chem.*, 30, 259 (1965).

C. Dosage and Dosage Delivery Forms

The type of compound and the carrier and the amount will vary widely depending on the species of the warm blooded animal or human, body weight, and tumor being treated. The dosage administered will vary depending upon known factors, such as the pharmacodynamic characteristics of the particular agent and its mode and route of administration, the age, health and weight of the recipient; the nature and extent of the symptoms; the kind of concurrent treatment; the frequency of treatment; and the effect desired.

The benzimidazole is preferably micronized or powdered so that it is more easily dispersed and solubilized by the body. Processes for grinding or pulverizing drugs are well known in the art. For example, a hammer mill or similar milling device are used. The preferred particle size is less than about 100 μ and preferably less than 50 μ .

The dosage administered will vary depending upon known factors such as the pharmacodynamic characteristics of the particular active ingredient, and its mode and route of administration; age, sex, health, metabolic rate, absorptive efficiency and/or weight of the recipient; nature and extent of symptoms; kind of concurrent treatment, frequency of treatment; and the effect desired.

A "tumor growth inhibiting amount" of the benzimidazole derivatives is that amount which is effective to inhibit or slow the growth of a tumor.

Dosage forms (compositions) suitable for internal administration contain from about 1.0 milligram to about 5000 milligrams of active ingredient per unit. In these pharmaceutical compositions, the active ingredient will ordinarily be present in an amount of about 0.5–95% by weight based on the total weight of the composition. Based on the body weight of the patient, the dosage may be administered in one or more doses several times per day or per week. Multiple dosage units may be required to achieve a therapeutically effective amount. For example, if the dosage form is 1000

mg, and the patient weighs 40 kg, one pill will provide a dose of 25 mg per kg for that patient. It will provide a dose of only 12.5 mg/kg for a 80 kg patient.

The compounds have shown dose responsiveness in vivo against viruses and cancers in mice at 500 mg/kg, 2500 mg/kg, 3500 mg/kg, 4000 mg/kg, 5000 mg/kg and 6000 mg/kg. Generally a dosage effective in mice translates to about 1/12 of the dosage required in humans. By way of general guidance, for humans a dosage of as little as about 30 milligrams (mg) per kilogram (kg) of body weight and up to about 10000 mg per kg of body weight is suitable. Preferably from 50 mg/kg to about 5000 mg/kg of body weight is used. Most preferably the doses are between 100 mg/kg to about 3000 mg/kg of body weight. However, a dosage of between about 2 milligrams (mg) per kilogram (kg) of body weight to about 400 mg per kg of body weight is also suitable for some indications.

Intravenously, the most preferred doses may range from about 1 to about 1000 mg/kg/minute during a constant rate infusion. Benzimidazole derivatives may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three, or four times daily. The benzimidazole derivatives are given in one or more doses on a daily basis or from one to three times a week.

The benzimidazole derivatives may also be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

Generally, the dosage in man is lower than for small warm blooded mammals such as mice. A dosage unit may comprise a single compound or mixtures thereof with other compounds or other cancer inhibiting compounds or tumor growth inhibiting compounds or anti-viral compounds. The dosage unit can also comprise diluents, extenders, carriers and the like. The unit may be in solid or gel form such as pills, tablets, capsules and the like or in liquid form suitable for oral, rectal, topical, intravenous injection or parenteral administration or injection into or around the tumor.

The benzimidazole derivatives are typically mixed with a pharmaceutically acceptable carrier. This carrier can be a solid or liquid and the type is generally chosen based on the type of administration being used. The active agent can be coadministered in the form of a tablet or capsule, as an agglomerated powder or in a liquid form. Examples of suitable solid carriers include lactose, sucrose, gelatin and agar. Capsule or tablets are easily formulated and can be made easy to swallow or chew, other solid forms include granules, and bulk powders. Tablets may contain suitable binders, lubricants, diluents, disintegrating agents, coloring agents, flavoring agents, flow-inducing agents, and melting agents. Examples of suitable liquid dosage forms include solutions or suspensions in water, pharmaceutically acceptable fats and oils, alcohols or other organic solvents, including esters, emulsions, syrups or elixirs, suspensions, solutions and/or suspensions reconstituted from non-effervescent granules and effervescent preparations reconstituted from effervescent granules. Such liquid dosage forms may contain, for example, suitable solvents, preservatives, emulsifying agents, suspending agents, diluents, sweeteners, thickeners, and melting agents. Oral dosage forms optionally contain flavorants and coloring agents. Parenteral and intravenous forms would also include

minerals and other materials to make them compatible with the type of injection or delivery system chosen.

D. Examples of Formulation

The benzimidazole derivatives of this invention are administered as treatment for cancer and viral infections, including retroviral, by any means that produces contact of the active agent with the agent's site of action in the body. The antitumor compounds (active ingredients) of this invention are administered to inhibit tumors by any means that produces contact of the active ingredient with the agent's site of action in the body of a mammal. They can be administered by any conventional means available for use in conjunction with pharmaceuticals, either as individual therapeutic agents or in a combination of therapeutic agents. They can be administered alone, but generally are administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

The benzimidazole derivatives are administered in oral dosage forms as tablets, capsules (each of which includes sustained release or timed release formulations), pills, powders, granules, elixirs, tinctures, suspensions, syrups, and emulsions. The benzimidazole derivatives may also be administered in intravenous (bolus or infusion), intraperitoneal, subcutaneous, or intramuscular form, all using dosage forms well known to those of ordinary skill in the pharmaceutical arts.

In the methods of the present invention, the compounds herein described in detail can form the active ingredient, and are typically administered in admixture with suitable pharmaceutical diluents, excipients, or carriers (collectively referred to herein as a pharmaceutically acceptable carrier or carrier materials) suitably selected with respect to the intended form of administration, that is, oral tablets, capsules, elixirs, syrups and the like, and consistent with conventional pharmaceutical practices.

For instance, for oral administration in the dosage unit form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic, pharmaceutically acceptable, inert carrier such as lactose, starch, sucrose, glucose, methyl cellulose, magnesium stearate, dicalcium phosphate, calcium sulfate, mannitol, sorbitol and the like.

For oral administration in liquid dosage form, the oral drug components are combined with any oral, non-toxic, pharmaceutically acceptable inert carrier such as ethanol, glycerol, water, and the like. Moreover, when desired or necessary, suitable binders, lubricants, disintegrating agents, and coloring agents can also be incorporated into the mixture. Suitable binders include starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth, or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes, and the like. Lubricants used in these dosage forms include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride, and the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum, and the like.

The benzimidazole derivatives can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles, and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine, or phosphatidylcholines.

Benzimidazole derivatives may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinylpyrrolidone, pyran copolymer, polyhydroxypropylmethacrylamide-phenol,

polyhydroxyethylaspartamidephenol, or polyethyleneoxide-polylysine substituted with palmitoyl residues. Furthermore, the compounds of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyglycolic acid, copolymers of polylactic and polyglycolic acid, polyepsilon caprolactone, polyhydroxy butyric acid, polyorthoesters, polyacetals, polydihydropyrans, polycyanoacrylates, and crosslinked or amphipathic block copolymers of hydrogels.

The active ingredient can be administered orally in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions. It can also be administered parentally, in sterile liquid dosage forms.

Gelatin capsules may contain the active ingredient and powdered carriers, such as lactose, starch, cellulose derivatives, magnesium stearate, stearic acid, and the like. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactured as sustained release products to provide for continuous release of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric coated for selective disintegration in the gastrointestinal tract.

Liquid dosage forms for oral administration can contain coloring and flavoring to increase patient acceptance. In general, water, a suitable oil, saline, aqueous dextrose (glucose), and related sugar solutions and glycols such as propylene glycol or polyethylene glycols are suitable carriers for parenteral solutions. Solutions for parenteral administration preferably contain a water soluble salt of the active ingredient, suitable stabilizing agents, and if necessary, buffer substances. Antioxidizing agents such as sodium bisulfite, sodium sulfite, or ascorbic acid, either alone or combined, are suitable stabilizing agents. Also used are citric acid and its salts and sodium EDTA. In addition, parenteral solutions can contain preservatives, such as benzalkonium chloride, methyl- or propyl-paraben, and chlorobutanol. Suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences, Mack Publishing Company, a standard reference text in this field.

Useful pharmaceutical dosage forms for administration of the compounds of this invention are illustrated as follows:

Capsules

A large number of unit capsules are prepared by filling standard two-piece hard gelatin capsules each with 100 to 500 milligrams of powdered active ingredient, 5-150 milligrams of lactose, 5-50 milligrams of cellulose, and 6 milligrams magnesium stearate.

Soft Gelatin Capsules

A mixture of active ingredient in a digestible oil such as soybean oil, cottonseed oil or olive oil is prepared and injected by means of a positive displacement pump into gelatin to form soft gelatin capsules containing 100-500 milligrams of the active ingredient. The capsules are washed and dried.

Tablets

A large number of tablets are prepared by conventional procedures so that the dosage unit was 100-500 milligrams of active ingredient, 0.2 milligrams of colloidal silicon dioxide, 5 milligrams of magnesium stearate, 50-275 milligrams of microcrystalline cellulose, 11 milligrams of starch and 98.8 milligrams of lactose. Appropriate coatings may be applied to increase palatability or delay absorption.

Injectable

A parenteral composition suitable for administration by injection is prepared by stirring 1.5% by weight of active

ingredient in 10% by volume propylene glycol and water. The solution is made isotonic with sodium chloride and sterilized.

Suspension

An aqueous suspension is prepared for oral administration so that each 5 ml contain 100 mg of finely divided active ingredient, 200 mg of sodium carboxymethyl cellulose, 5 mg of sodium benzoate, 1.0 g of sorbitol solution, U.S.P. and 0.025 ml of vanillin.

The present invention also includes pharmaceutical kits useful, for example, for the treatment of HIV infection, which comprise one or more containers containing a pharmaceutical composition comprising a therapeutically effective amount of a benzimidazole derivative. Such kits may further include, if desired, one or more of various conventional pharmaceutical kit components, such as, for example, containers with one or more pharmaceutically acceptable carriers, additional containers, etc., as will be readily apparent to those skilled in the art. Printed instructions, either as inserts or as labels, indicating quantities of the components to be administered, guidelines for administration, and/or guidelines for mixing the components, may also be included in the kit. In the present disclosure it should be understood that the specified materials and conditions are important in practicing the invention but that unspecified materials and conditions are not excluded so long as they do not prevent the benefits of the invention from being realized.

The following examples are illustrative and are not meant to be limiting to the invention.

Colon, Breast and Lung Tumor Cells Test

The following cell culture tests were performed to test the toxicity of the benzimidazole compounds on colon, breast and lung human tumor cells. The viability of the cells were tested by looking at MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction. MTT assay is a well known measure of cell viability.

The colon tumor cells H29 from American Type Culture Collection (ATCC) and the breast cells (MX1 from cell lines from ATCC) were cultured in Eagle's Minimal Essential Medium with 10% fetal bovine serum. The lung tumor cells (A549 from ATCC cell lines) were cultured in Ham's F12 medium with 10% fetal bovine serum.

The tumor cells were passaged and seeded into culture flasks at the desired cell densities. The culture medium was decanted and the cell sheets were washed twice with phosphate buffered saline (PBS). The cells were trypsinized and triturated prior to seeding the flasks. Unless otherwise indicated the cultures were incubated at $37 \pm 1^\circ \text{C}$. in a humidified atmosphere of $5 \pm 1\%$ carbon dioxide in air. The cultures were incubated until they were 50–80% confluent.

The cells were subcultured when the flasks were subconfluent. The medium was aspirated from the flasks and the cell sheets rinsed twice with PBS. Next, the Trypsin Solution was added to each flask to cover the cell sheet. The Trypsin Solution was removed after 30–60 seconds and the flasks were incubated at room temperature for two to six minutes. When 90% of the cells became dislodged, growth medium was added. The cells were removed by trituration and transferred to a sterile centrifuge tube. The concentration of cells in the suspension was determined, and an appropriate dilution was made to obtain a density of 5000 cells/ml. The cells were subcultured into the designated wells of the 96-well bioassay plates (200 microliter cell suspension per well). PBS was added to all the remaining wells to maintain humidity. The plates were then incubated overnight before test article treatment.

Each dose of test article was tested by treating quadruplicate wells of cultures with 100 microliter of each dilution.

Those wells designated as solvent controls received an additional 100 microliter of methanol control; negative controls wells received an additional 100 microliters of treatment medium. PBS was added to the remaining wells not treated with test article or medium. The plates were then incubated for approximately 5 days.

At the end of the 5 day incubation, each dose group was examined microscopically to assess toxicity. A 0.5 mg/ml dilution of MET was made in treatment medium, and the dilution was filtered through a 0.45 micrometer filter to remove undissolved crystals. The medium was decanted from the wells of the bioassay plates. Immediately thereafter, 2000 microliter of the filtered MTT solution was added to all test wells except for the two untreated blank test wells. The two blank wells received 200 microliters of treatment medium. The plates were returned to the incubator for about 3 hours. After incubation, the MTT containing medium was decanted. Excess medium was added to each well and the plates were shaken at room temperature for about 2 hours.

The absorbance at 550 nm (OD_{550}) of each well was measured with a Molecular Devices (Menlo Park, Calif.) VMax plate reader.

The mean OD_{550} of the solvent control wells and that of each test article dilution, and that of each of the blank wells and the positive control were calculated. The mean OD_{550} of the blank wells was subtracted from the mean of the solvent control wells, and test article wells, respectively to give the corresponding mean OD_{550} .

$$\% \text{ of Control} = \frac{\text{corrected mean } \text{OD}_{550} \text{ of Test Article Dilution}}{\text{corrected mean of } \text{OD}_{550} \text{ of Solvent Control}} \times 100$$

Dose response curves were prepared as semi-log plots with % of control on the ordinate (linear) and the test article concentration on the abscissa (logarithmic). The EC_{50} was interpolated from the plots for each test article.

For the test articles administered in methanol, separate responses were prepared to correct for the methanol data.

Adriamycin was used as a positive control. In all cases, it was more toxic than any of the test materials by one or two logs. Adriamycin is one of the more potent agents in current use and one with significant side effects. The peak plasma concentration of other, quite effective chemotherapeutic agents may be 10 to 50 times higher than that of Adriamycin.

The EC_{50} is the concentration at which one half of the cells are killed.

TABLE 1

Test Material	EC-50 Result (ppm)					
	HT29	HT29	MX1	MX1	A549	A549
Adriamycin	0.03	0.006	0.02	0.001	0.03	0.009
benomyl	0.742	0.747	1.42	2.42	0.980	1.02
carbendazim	0.621	0.662	0.829	0.856	0.956	0.836

In normal healthy cells, the following results were obtained. As is evident, the benomyl and carbendazim were much less toxic to normal healthy cells than adriamycin.

TABLE 2

Test Material	EC-50 Broncheal Cells		Keratinocyte Cells		Fibroblasts	
Benomyl	0.728	0.682	3.25	2.4	3.24	2.81
Carbendazim	0.320	0.506	0.752	0.822	1.52	1.42
Adriamycin	0.015	0.0020	0.0035	0.0093	0.065	0.10

In a related study using lung tumor cells (A-549) breast tumor cells (MCF-7) and colon tumor cells (HT-29), thia-bendazole effectively killed these cells. Table 3 summarizes the results

TABLE 3

Concentration (ppm)	Optical Density		
	A-549	MCF-7	HT-29
0-Control	0.600	0.245	0.398
173	0.007	0.007	0.005
35	0.411	0.025	0.011
17.3	0.851	0.258	0.204
3.46	1.12	0.466	0.713
0.87	1.32	0.507	0.852

These experiments show that these compositions are effective in killing tumor cells of the breast, colon and lung type.

Carbendazim has shown broad-scale efficacy against multiple cancer types both in vitro and in vivo. The cancers tested include colon, lung, breast, prostate, pancreatic, leukemia, melanoma, neuroblastoma, ovarian, neck and head, and brain. Also multiple cell lines were tested in almost instances.

The initial efficacy is comparable to existing best available drugs. But with carbendazim, the tumors do not recur or reappear as happens with Cytoxan and Taxol, which otherwise are quite good against breast cancer. Similarly, pancreatic cancer does not appear to come back as often happens with Gemcitabine treatment.

Carbendazim is particularly good in mouse melanoma in mice, which many people believe is the best predictive model for efficacy in humans. It has shown outstanding broad and good results in the human tissue cloning test. This is an in vitro test on conventionally treated and recently excised human tumors.

Carbendazim is equally effective against p53 deficient/defective cell lines, unlike most existing drugs. It appears that carbendazim induces apoptosis in cancer cells at sub-lethal concentrations to normal cells.

Other benefits of carbendazim are:

Its oral LD₅₀ in mice is quite high (11,000 mg/kg), a low overall toxicity unlike most cancer drugs. For perspective the LD₅₀ of table salt is 3750 mg/kg.

It is effective in cancers that form tumors and those that do not, e.g. both carcinomas and sarcomas.

The results of these studies are provided in more detail below.

Mechanism of Action Studies

Some of the pharmacological effects of carbendazim were demonstrated by studying its ability to induce apoptosis in cancer cells, studying its effect on p53-abnormal cell lines and determining during which cellular life cycle phase carbendazim exerts its effects.

Apoptosis Study:

Apoptosis is a specific type of cell death which differs from necrosis and is characterized by specific

morphological, biochemical and micellular cell changes. Abnormalities in p53 expression are generally linked with the prevention of apoptosis and p53 abnormalities are common in human tumors which are resistant to conventional cytotoxic agents.

Summary of test results: The extent of apoptosis is measured in human tumor cell lines after treatment for 1, 2, 3 and 4 days with carbendazim. At each of these time points, the cells are harvested and assayed using the terminal deoxynucleotidyl transferase (TdT) assays. Both microscopy and flow cytometry were used for the TdT assay.

In MCF7, HT29, B16 and SK-MES cell lines, there was a concentration dependent effect on cell growth. In most cases at concentrations of carbendazim greater than 1 μ g/ml, the growth rate was significantly slower than in the untreated samples. After completion of the studies on the MCF7 and HT29 cell lines, it was evident that the 0.1 μ g/ml concentration had little effect on either apoptosis or cell growth. Therefore subsequent assays were with 5 μ g/ml instead of 0.1 μ g/ml.

The growth of MCF7 (breast cancer) cells was not significantly affected by carbendazim below 10 μ g/ml as shown by either method. However, at 10 μ g/ml the increase in apoptosis was evident at days 3 and 4. The increase in apoptosis was low, less than 10% at the high concentration. After normalization for the cell growth, carbendazim at 10 μ g/ml had an effect at day 4.

The growth of HT 29 (colon) cancer cells was not largely slowed down by carbendazim below 10 μ g/ml. Concentration-dependent increase in apoptosis was observed at days 3 and 4, reaching >25% apoptosis in the presence of 10 μ g/ml carbendazim. After normalization for the cell growth rates, the concentration-response effect was seen at day 4.

At all concentrations, carbendazim affected the growth rates in B16 murine melanoma cell line. Some concentration dependent effects on apoptosis were seen at days 1-4 by TdT microscopy and days 2-4 by flow cytometry. The concentration effects at days 14 were much more evident after normalization of the apoptosis for the growth rates.

The growth of SK-MES cells was slowed down by all concentrations of carbendazim past day 1. The percent of apoptosis showed a concentration-response effect at days 1-4 by microscopic TdT assay and by flow cytometry at days 3 and 4. A normalized graph showed a concentration response effect of the compound on apoptosis at all days.

Conclusion: At concentrations less than 10 μ g/ml, the greatest response to carbendazim was seen in the SK-MES lines (lung), followed by B16 (murine melanoma), HT29 (colon), and MCF7 (breast) cells. The HT29, SK-MES and B16 cell lines express abnormal p53. Accordingly carbendazim can induce apoptosis in p53 abnormal cell lines.

Selectivity in killing p53 abnormal cell lines

Carbendazim provides in vivo activity against HT29 tumor cells which express abnormal p53.

Summary of test results: Pairs of tumors of the same type were chosen, one expressing normal p53 and the other abnormal p53. Breast lines used were MCF7 for the normal p53, V4B, a MCF7 cells transformed with an empty vector, and VM4K, an MCF7 cells transfected with a vector encoding abnormal p53. In the colorectal cancer model HCT116 with a normal p53 was used and DLD-1 was used as the abnormal p53 cell line. The tumor cells were grown the presence and absence of carbendazim for 7 days. Cell growth rates were determined in each group by counting the cell numbers daily using a Coulter counter.

In the breast model at day 7, using 1 μ g/ml of carbendazim, cell counts in all three cell lines were between

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50% and 60% of the control. At concentrations of 5 and 10 $\mu\text{g/ml}$ the MCF7 cell counts were 45% and 36% of the control. The V4B and VM4K cell lines were less than 10% of the respective controls. The data from the VM4K cell line would also indicate the carbendazim is again selectively killing the p53 abnormal cells, however, there was also a marked decrease in the cell numbers in the V4B line carrying only the empty vector. In light of the controversy regarding the p53 status in MCF7 cells, which some researchers claim to express abnormal p53, these differences may be more difficult to interpret.

At day 7 in the colorectal model, at 1 $\mu\text{g/ml}$ concentration carbendazim cell counts in the DLD1 line (abnormal p53) were only 34% of the control. The HCT 116 cell line, (normal p53) showed cell counts that were 78% of the control suggesting that carbendazim may selectively kill p53 abnormal cells. At carbendazim concentrations of 5 and 10 $\mu\text{g/ml}$, cell counts were less than 10% of the control in both the DLD-1 and HCT116 lines indicating that at the larger concentration, the drug was equally toxic to cell lines containing abnormal and normal p53.

Conclusion: At higher concentrations, 5 and 10 $\mu\text{g/ml}$, the drug was equally toxic to cell lines containing abnormal p53 and normal p53 in both colon and breast cancers.

In Vitro Studies of Carbendazim on Cancer Cell Lines

A dose response effect of carbendazim in a human tumor cloning forming units study HCU) is summarized below. This is an in vitro test of treatments on conventionally treated, then recently excised human tumors. It is an important study because carbendazim is showing effectiveness against cell lines which have survived conventional treatment and which themselves have not had undergone too many passages (due to recent excision). This is significant since long living cell lines undergo changes (passages), some of which may affect their resistance to some drugs, and chemotherapeutic agent resistant cells can be formed. The data show the activity as the tumors tested that had $\leq 50\%$ survival (a high number is desirable).

In Vitro Human Tumor Colony Forming Units Test

Solid tumors removed by patients are minced into 2 to 5 mm fragments and immediately placed in McCoy's Medium SA plus 10% heat inactivated newborn calf serum plus 1% penicillin/streptomycin. Within 4 hours, these solid tumors are mechanically dissociated with scissors, forced through No. 100 stainless steel mesh, through 25 gauge needles, and then washed with McCoy's medium as described above. Ascitic, pleural, pericardial fluids and bone marrow are obtained by standard techniques. The fluid or marrow is placed in sterile containers containing 10 units of preservative free heparin per ml. of malignant fluid or marrow. After centrifugation at 150xg for 10 minutes, the cells are harvested and washed with McCoy's medium plus 10% heat inactivated calf serum. The viability of cell suspensions is determined on a hemocytometer with trypan blue.

Cells to be cloned are suspended in 0.3% agar in enriched CMRL1066 supplemented with 15% heat inactivated horse serum, penicillin (100 units/ml), streptomycin (2 mm), glutamine (2 mM), insulin (3 units/ml), asparagine (0.6 mg/ml), and HEPES buffer (2 mM). For the continuous exposure test each compound is added to the above mixture. Cells are placed in 35 mm petri dishes in a top layer of agar over an underlayer of agar to prevent growth of fibroblasts. Three plates are prepared for each data point. The plates are placed in a 37° C. incubator, and are removed on day 14 for counting of the number of colonies in each plate. The number of colonies (defined as 50 cells) formed in the 3

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compound treated plates is compared to the number of colonies formed in the 3 control plates, and the percent colonies surviving at the concentration of compound can be estimated. Three positive control plates are used to determine survival rate. Orthosodium vanadate at 200 $\mu\text{g/ml}$ is used as the positive control. If there is $<30\%$ colonies in the positive control when compared to the untreated control, the test is evaluated.

Activity of Carbendazim Against Human Tumor Colony Forming Units							
Tumor Type	1 Hour Exposure Concentration - $\mu\text{g/ml}$			1 Hour Exposure Concentration - $\mu\text{g/ml}$			
	0.5	5.0	50	0.5	5.0	10	50
Brain	—	—	—	0/2	0/2	—	2/2
Breast	0/1	0/1	0/1	0/3	0/4	0/1	2/3
Cervix	—	—	—	0/1	0/1	—	1/1
Colon	0/1	0/1	0/1	0/5	1/5	—	2/5
Head & Neck	0/1	0/1	0/1	0/1	0/1	—	1/1
Kidney	0/1	0/1	0/1	0/2	0/2	—	1/2
Lung, non-small cell	0/1	0/1	0/1	0/6	2/6	0/1	2/5
Melanoma	0/2	0/2	1/2	0/3	1/3	—	2/3
Mesothelioma	—	—	—	0/1	0/1	0/1	—
Ovary	0/3	0/3	0/3	0/1	2/13	—	10/13
Sarcoma	0/1	0/1	1/1	0/1	0/1	—	1/1
Stomach	—	—	—	0/1	0/1	—	1/1
Uterus	—	—	—	0/3	0/3	—	2/3
Unknown Primary	—	—	—	0/1	0/1	—	0/1
	0/11	0/11	2/11	0/4	6/44	0/3	27/41
	0%	0%	18%	0%	14%	0%	66%

(4-thiazolyl)-1H-benzimidazole shows efficacy in the Human Tumor Colony Forming Units test described above when tested using continuous exposure of the cells to the (4-thiazolyl)-1H-benzimidazole.

The following table summarizes these results showing positive results on a number of cancer types:

Compound	1 Hour Exposure Concentration - $\mu\text{g/ml}$			Continuous Exposure Concentration - $\mu\text{g/ml}$		
	0.5	5.0	50.0	0.5	5.0	50.0
2-(methoxycarbonylamino) benzimidazole	0/1	0/1	0/1	0/14	3/14	5/14
(4-thiazolyl)-1H-benzimidazole	0/3	0/3	0/3	1/10	2/10	7/10

In Vivo Studies of Carbendazim on Cancer Cell Lines

The dose response effect of carbendazim in mice infected with various cancer types was studied in standard screening tests.

The results of these studies conducted to study the dosage response of carbendazim on various cancer types are summarized below. These results are representative of a number of tests in which a known chemotherapeutic agent is used as the control so that the efficacy of the carbendazim can be compared to it. Efficacy of carbendazim has been shown in the following cancers:

In a Prostate cancer model doses of 4000, 5000 and 6000 (mg/kg given once weekly) were as effective as Mitoxantrone at 70 days. It was better than Cytosin at these doses

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of 4000, 5000 and 6000 (mg/kg given twice weekly) through 40 days. The tables show tumor weight in mg for each dose. The carbendazim was given once a week (p.o) and the Mitoxantrone was given by i.v. (q.d.x5).

dose	day 1	day 5	day 9	day 12	day 16	day 19
peanut oil control	61.6	88.9	146.8	184.9	278.1	305.8
6000 mg/kg carbendazim	62.1	92.2	140.4	162	226.5	275.99
5000 mg/kg carbendazim	63.1	100.1	116.8	138.3	280.4	246.9
4000 mg/kg carbendazim	63.1	97.5	159	192.7	282.1	311.6
Mitoxantrone 1.5 mg/kg	61.9	91.5	120.4	150.9	169.7	187.1
dose	day 23	day 26	day 30	day 33	day 37	day 40
peanut oil control	385.6	592.4	518	625.2	537.6	594
6000 mg/kg carbendazim	301.9	400.9	416.6	447.3	546.3	514.4
5000 mg/kg carbendazim	281.3	374.6	370.6	428.6	406.4	391.6
4000 mg/kg carbendazim	316.6	368.1	351.3	410.7	506.8	484.9
Mitoxantrone 1.5 mg/kg	208.5	248	247.3	296.9	363	465
dose	day 44	day 47	day 51	day 53	day 59	day 61
peanut oil control	714.1	777.4	665.7	764.8	981.3	936
6000 mg/kg carbendazim	505.2	484	438.3	499.8	492.2	480.1
5000 mg/kg carbendazim	445.7	454.7	505.9	543.3	628.6	579.1
4000 mg/kg carbendazim	481.5	511.5	543.1	552.9	507.8	560.3
Mitoxantrone 1.5 mg/kg	545.6	474.9	495.8	566.2	656.8	657.4
dose				day 65	day 68	day 72
peanut oil control				—	—	—
6000 mg/kg carbendazim				581.9	525.9	667.3
5000 mg/kg carbendazim				562.3	562.3	602
4000 mg/kg carbendazim				631.2	697.8	739.1
Mitoxantrone 1.5 mg/kg				775.1	820.8	707

Both the Cytosin and the carbendazim were given p.o. twice a week

dose	day 1	day 5	day 8	day 12	day 15	day 19
peanut oil control	66.9	118.2	185.8	250.2	264.5	351
6000 mg/kg carbendazim	66.7	97.3	143	193.5	237.9	316.2
5000 mg/kg carbendazim	67.9	84.9	126.8	152.8	184.2	199.5
4000 mg/kg carbendazim	67.2	110.4	157.6	192.6	238.9	298.7
Cytosin 300 mg/kg	66.7	98.4	179.4	234.6	259.7	278.1
dose	day 22	day 26	day 29	day 33	day 36	day 39
peanut oil control	416.1	446.2	555.3	802.7	868.4	1032.3
6000 mg/kg carbendazim	331.5	371.7	421.7	517.2	529.9	595.2

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5000 mg/kg carbendazim	236.8	247.6	293.6	351.5	409.8	497.8
4000 mg/kg carbendazim	330.5	347.9	346.6	421.1	464.9	517
Cytosin 300 mg/kg	351.2	467.8	583.8	786.1	904.2	1143.5

10 In the Colon—HT29 mouse model carbendazim at doses of 4000, 5000 and 6000 (mg/kg given twice weekly) it is better than Cytosin in this model. At a dose of 3000 (mg/kg given twice weekly) it is also better than Cytosin.

The carbendazim and Cytosin were given twice weekly.

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	dose	day 1	day 5	day 8	day 12	day 16	day 19
20	peanut oil control	53.5	67.1	98.6	154.6	187	236.3
	Cytoxan 300 mg/kg	51.3	60.3	42	58.5	64	87.8
	6000 mg/kg carbendazim	53.5	55.8	45.2	62.8	46.4	59.4
	5000 mg/kg carbendazim	53.5	58	58	104.3	83.2	109.7
	4000 mg/kg carbendazim	51.3	63.8	59.7	85.8	81.1	119.4
25							
	dose	day 23	day 26	day 29	day 37	day 40	
30	peanut oil control	335.5	433.1	499.9	786.3	984.9	
	Cytoxan 300 mg/kg	137.7	174	252	407.3	503	
	6000 mg/kg carbendazim	77	78.4	60	51.3	62.5	
	5000 mg/kg carbendazim	129.2	149.4	133.3	185.6	185.1	
	4000 mg/kg carbendazim	142.1	159.6	156.2	184.6	212.9	
35							
	dose	day 1	day 6	day 9	day 13	day 16	day 20
40	control- no treatment	77.1	172.3	231.4	348.1	409.3	478.4
	peanut oil control	75.8	172.2	218.2	300.3	344	460
	Cytoxan 300 mg/kg	76.6	132.5	152.5	142.8	188.1	266
	3000 mg/kg carbendazim	75.8	108.1	110.4	141.4	152.3	121.7
		dose				day 23	day 26
45	control- no treatment				582.8	710.2	867.3
	peanut oil control				540.8	701.5	863
	Cytoxan 300 mg/kg				372.7	375	478
	3000 mg/kg carbendazim				141.8	173.5	209.5

50 In the Breast—MX-1 model, carbendazim at 4000, 5000, and 6000 (mg/kg, twice weekly) was dose responsive in slowing the growth of the tumor and was better than Cytosin. It is also very effective in MCF-7L breast line. At a dose of 3000, (mg/kg given twice weekly) it was equivalent to Navelbine.

55 The Cytosin and the carbendazim were given twice weekly, p.o.

dose	day 1	day 5	day 8	day 12	day 15
peanut oil control	70.9	208.6	526	1153.6	2267.9
Cytosin 300 mg/kg	70	32.9	4.2	3.2	0
6000 mg/kg carbendazim	70.4	151.8	259.9	492.9	663.6
5000 mg/kg carbendazim	70.1	157.4	272.1	535.9	856.4
4000 mg/kg carbendazim	70.3	158	320.4	626.2	1126.5

65 When the tumor was shrunk with Taxol first and then the carbendazim therapy started when the tumor began to grow

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again on day 130, the carbendazim treatment was begun and the tumor subsequently shrunk to zero. The Navelbine was given 1.6 mg./kg, qdx5, i.p.; the Taxol was given 16 mg/kg, qdx5, i.p.; and the carbendazim was dosed twice weekly, p.o.. The carbendazim treatment was better than Navelbine and as good as Taxol in this study.

dose		day 1	day 5	day 8	day 12	day 15	
peanut oil control		72.9	91.4	95.2	117.1	121.4	
3000 mg/kg carbendazim		70.8	98.7	90.8	110.1	106.8	
Navelbine		68.1	95.3	83.2	106.1	116.3	
Taxol		68.9	85.2	52.1	36.3	27.5	
dose		day 20	day 23	day 26	day 29	day 35	day 37
peanut oil control		157.6	171.9	170.1	211.6	226.1	229.4
3000 mg/kg carbendazim		112.5	137.4	127.9	135.2	140.1	137.9
Navelbine		135.3	160.5	157.9	162.8	195.8	212.2
Taxol		28.5	31.8	32.4	34.2	36.3	32.9
dose		day 41	day 44	day 48	day 51	day 55	day 58
peanut oil control		253.2	257.7	252.1	247.8	263.9	278.3
3000 mg/kg carbendazim		129.2	128	134.6	111.9	98.7	107.9
Navelbine		223.1	217.4	237.3	222.3	255.8	251.6
Taxol		29.9	38.7	29.7	33.3	27.1	35.2
dose		day 62	day 65	day 69	day 72	day 76	day 79
peanut oil control		274.4	252.8	275.2	277.9	274.7	296.4
3000 mg/kg carbendazim		102.3	103.7	85.1	81.4	75.2	66.1
Navelbine		254.2	257.3	306.1	301.9	307.6	340.7
Taxol		35.2	35.2	22.2	33.2	32.9	35.2
dose		day 82	day 85	day 89	day 93	day 97	day 100
peanut oil control		277.2	276.1	168.7	197.2	278.8	264.6
3000 mg/kg carbendazim		66.3	61.9	18.1	39.3	53.9	54.2
Navelbine		322	352.1	249.9	314.9	375.6	368.6
Taxol		35.2	39.2	17	36.8	43.7	46.8
dose		day 103	day 105	day 110	day 113	day 116	day 119
peanut oil control		266.1	263.1	277.2	278.3	288.9	305.3
3000 mg/kg carbendazim		49.7	52.4	52.4	49.9	51.3	43.7
Navelbine		399.3	391.1	418.8	440.6	544.4	491.9
Taxol		53	53.2	50.5	48.4	65.9	65.8
dose		day 124	day 131	day 134	day 137	day 140	day 144
peanut oil control		331	371.2	396.7	440.5	449.5	482.8
3000 mg/kg carbendazim		43.7	49.2	47.1	53.9	49.4	52.2
Navelbine		514.9	607.3	741.5	692.6	687.4	772.3
Taxol		76.6	86.8	92.8	97.9	92.6	92.4
dose		day 147	day 152	day 155	day 158	day 161	day 165
peanut oil control		506.6	540.7	—	—	—	—
3000 mg/kg carbendazim		50.6	53.9	49.4	40.7	49.4	49.4
Navelbine		811	809.4	—	—	—	—
Taxol		104.6	105.9	116	68	73.1	68

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	dose	day 172	day 175	day 179	day 182	day 186	day 189
5	peanut oil control						
	3000 mg/kg carbendazim	61.2	64.6	69.6	66.3	74.1	81.3
	Navelbine						
	Taxol	68	71.9	73.1	68.1	67.1	68.1
10	dose	day 193	day 196	day 200	day 207	day 210	day 214
	peanut oil control						
	3000 mg/kg carbendazim	79.6	92.2	96.7	117.3	126.2	137
15	carbendazim						
	Navelbine						
	Taxol	66.3	63.9	58.5	51.4	54.8	34.1
	dose	day 217	day 221	day 224	day 228	day 232	day 236
20	peanut oil control						
	3000 mg/kg carbendazim	142.1	162.3	167.3	175.8	209.3	145.7
	Navelbine						
	Taxol	27.5	20.8	20.8	20.8	0	0
25	dose				day 239	day 245	day 249
	peanut oil control						
	3000 mg/kg carbendazim				136.3	158.7	197.1
	Navelbine						
	Taxol				0	0	0
30							
<p>In a third study carbendazim was tested in mice MX-1 model with the mice receiving estrogen tablets. Estrogen accelerates the growth of the breast cancer. At doses of 2000, 4000 and 6000 (mg/kg given once weekly) it was better than both Navelbine (1.6 mg/kg, qdx5, i.p.) and Taxol (16 mg/kg, qdx5, i.p.) in this faster growing MX-1 cancer.</p>							
40	dose	day 1	day 5	day 9	day 12	day 16	
	peanut oil control	109.2	307.7	947.6	1702.8	3359.8	
	6000 mg/kg carbendazim	109.5	292.7	593.1	1279.6	1261.6	
	4000 mg/kg carbendazim	109.5	337.1	664.2	1143.3	1501.5	
45	3000 mg/kg carbendazim	110.4	312.4	603.6	1068	1502.8	
	2000 mg/kg carbendazim	110.4	342.3	752.3	1447.6	1609.1	
	Navelbine	110.2	278.6	874.5	1528.5	2746.8	
	Taxol	110.4	292.7	484.9	876.6	1941.5	
50							
<p>In the Pancreas (Mia-PaCa) model, carbendazim at 3000 and 4000 (mg/kg, twice weekly) is as good as or better than gemcitabine. At 2000 mg/kg the carbendazim was not as effective after 21 days. Gemcitabine was given on days 1,4,7 and 10 i.p.; the carbendazim was given p.o. twice weekly.</p>							
55	dose	day 1	day 5	day 9	day 12	day 16	
	peanut oil control	63.1	118.5	186.6	228.4	294.6	
60	4000 mg/kg carbendazim	64	78.9	121.3	113.4	133.4	
	3000 mg/kg carbendazim	63.1	71.8	100.1	100.4	139.6	
	2000 mg/kg carbendazim	63.7	85.2	128.4	155.1	213.4	
	Gemcitabine - 80 mg/kg	63.9	71.7	81.7	77.1	94.9	
	dose	day 19	day 23	day 26	day 30	day 33	day 37
65	peanut oil control	325.9	462.8	489.5	546.6		

In a third study carbendazim was tested in mice MX-1 model with the mice receiving estrogen tablets. Estrogen accelerates the growth of the breast cancer. At doses of 2000, 4000 and 6000 (mg/kg given once weekly) it was better than both Navelbine (1.6 mg/kg, qdx5, i.p.) and Taxol (16 mg/kg, qdx5, i.p.) in this faster growing MX-1 cancer.

In the Pancreas (Mia-PaCa) model, carbendazim at 3000 and 4000 (mg/kg, twice weekly) is as good as or better than gemcitabine. At 2000 mg/kg the carbendazim was not as effective after 21 days. Gemcitabine was given on days 1,4,7 and 10 i.p.; the carbendazim was given p.o. twice weekly.

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4000 mg/kg carbendazim	119.4	157	154.2	124.3	131.4	129.2
3000 mg/kg carbendazim	131.9	146.6	131.9	140.1	135.1	110.6
2000 mg/kg carbendazim	182.2	185	189.9	214.1	206.7	217.6
Gemcitabine - 80 mg/kg	111.3	167.1	204	258.7	330.3	404.3
dose	day 40	day 44	day 47	day 51	day 54	day 61
peanut oil control						
4000 mg/kg carbendazim						
3000 mg/kg carbendazim	105	105	120.1	124.1	124.1	118.6
2000 mg/kg carbendazim	214.55	214.5	210.9	222.4	225.6	214.1
Gemcitabine - 80 mg/kg	503.7					
dose	day 65	day 68	day 72	day 75	day 79	
peanut oil control						
4000 mg/kg carbendazim						
3000 mg/kg carbendazim	130	130	69.2	52.5	75.8	
2000 mg/kg carbendazim	217.3	217.3	202.6	178.2	169.7	
Gemcitabine - 80 mg/kg						

In the Panc-01 model for pancreatic cancer carbendazim at a dose of 5000 mg/kg was better than Gemcitabine at 32 days. The Gemcitabine is given i.p., q3dx4 and the carbendazim is given p.o. twice weekly to the end.

dose	day 1	day 4	day 8	day 11	day 18
control- no treatment	64.1	110.8	201.5	339.7	726
peanut oil control	64.3	123.8	200.3	306	740.3
Gemcitabine 80 mg/kg	64	106.3	171.7	248.5	561.4
5000 mg/kg carbendazim	64.4	115.5	166.8	247	417.9
dose	day 22	day 25	day 29	day 32	
control- no treatment	1001.4	1183			
peanut oil control	1174.1	1126.7			
Gemcitabine 80 mg/kg	943.6	1053	1183		
5000 mg/kg carbendazim	574.3	695.8	845	807.9	

In the Neuroblastoma (SK-N-MC) model a decrease in tumor growth at 5000 (mg/kg) is shown and there is favorable activity early in the study compared to topotecan. It is expected, based on learning from the other studies, that a lower dose would be effective. The topotecan is given i.p. qdx5 and the carbendazim is given p.o. twice weekly.

dose	day 1	day 5	day 9	day 12	day 16
peanut oil control	454.8	1034.1	1533.1	1141.8	1402
5000 mg/kg carbendazim	474.4	505.8	799.3	841.8	732
Topotecan 3 mg/kg	504.9	209.8	57.5	71.8	144.7
dose	day 19	day 23	day 25	day 31	
peanut oil control	1668.2	1852.8	2025		
5000 mg/kg carbendazim	1424.3	1480.5	936		
Topotecan 3 mg/kg	294.6	648.1	886.6	1150.2	

In the Rhabdomyosarcoma model, a childhood cancer, at day 29 carbendazim at doses of 3000, 4000 and 5000 (mg/kg

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given once weekly, p.o.) is quite effective against this tumor with no toxicity in the 3000 (mg/kg) group. Topotecan (i.p., qdx5) is quite active against this model.

dose	day 1	day 5	day 8	day 12	day 15
peanut oil control	58.1	121.3	177	223	340.9
5000 mg/kg carbendazim	58.1	95.1	87	88.1	106.4
4000 mg/kg carbendazim	59.5	110.7	124.2	121.6	155.3
3000 mg/kg carbendazim	58.2	110.4	136.6	176.7	248.2
Topotecan 3 mg/kg	58.3	82	53.3	24.1	26.6

dose	day 19	day 22	day 26	day 29	day 35	day 37
peanut oil control	558	689.3	894	948.3	1038.2	1098.5
4000 mg/kg carbendazim	112.6	107.9	137.5	174.3	238.8	317.4
3000 mg/kg carbendazim	113	127	140.8	144.3	131.9	152.4
2000 mg/kg carbendazim	313.1	291.2	384.3	417.4	591.2	492.9
Topotecan 3 mg/kg	13.4	18.9	40.9	64.2	107.9	158.9

dose	day 40	day 43	day 47
peanut oil control			
4000 mg/kg carbendazim	313.9	423	171.5
3000 mg/kg carbendazim	142.6	195.5	235.8
2000 mg/kg carbendazim	555.6	682.1	854.3
Topotecan 3 mg/kg	184.4	279.2	351.9

In the Lung cancer model MV522 all groups received Taxol and then received treatments shown starting 10 days later. At doses of 2000, 3000, 4000 and 5000 (mg/kg given twice weekly, p.o.) tumor growth was suppressed in a dose response and the tumor was shrunk in the 5000 mg/kg group. Day 1 is the start of the treatment with carbendazim.

dose	day 1	day 4	day 8	day 11	day 15	day 18
control - no treatment	6.6	5.6	7.3	18.3	39.6	80.2
peanut oil control	6.7	16.9	29.7	50.6	84.9	140.6
5000 mg/kg carbendazim	6.5	7.1	16.1	12.7	18.8	27.2
4000 mg/kg carbendazim	6.6	8.5	17.6	16.2	20.7	26.6
3000 mg/kg carbendazim	6.5	7.1	11.9	9.2	14.1	16
2000 mg/kg carbendazim	6.5	10.3	19.9	33.8	44.8	40.5
dose	day 22	day 25	day 29	day 32	day 36	
control - no treatment	128.8	180.1	270.7	307.3	594.6	
peanut oil control	233.9	341.4	488.7			
5000 mg/kg carbendazim	21.5	31.4	40.5	29.5	54	
4000 mg/kg carbendazim	40.8	39.7	58.9	47.3	62.5	
3000 mg/kg carbendazim	19.9	23.4	37.5	28.9	44.5	
2000 mg/kg carbendazim	37.9	43.5	49.8	41.1	53	
dose	day 39	day 42	day 46	day 49	day 52	day 56
control - no treatment	572.4	670.1				
peanut oil control						
5000 mg/kg carbendazim	63	86.8	86	1	0.5	
4000 mg/kg carbendazim	62.5	32				

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3000 mg/kg carbendazim	59.2	69.3	72.6	160.7	207.1	267.4
2000 mg/kg carbendazim	42.9	47.1	60.3	107.4	107.4	175.3
dose			day 59	day 63	day 66	
control - no treatment						
peanut oil control						
5000 mg/kg carbendazim						
4000 mg/kg carbendazim						
3000 mg/kg carbendazim			75.4	77		94.5
2000 mg/kg carbendazim			214.9	193.7		188.9

In a Medulloblastoma model (IMR32) a dosage dependent effect is seen with carbendazim at doses of 5000, 4000, and 3000 (mg/kg, p.o. twice weekly). It is compared with Topotecan (i.p. qdx5).

dose	day 1	day 7	day 9	day 12	day 15	day 19
peanut oil control	57.6	82.1	103.8	107.5	143.1	182.7
5000 mg/kg carbendazim	57.2	74.9	86.4	94.6	102	125.4
4000 mg/kg carbendazim	58.1	89.3	108	112.9	152.9	162.3
3000 mg/kg carbendazim	58.1	73.8	99.3	105.1	119.7	153.4
Topotecan 3 mg/kg	57.8	22.6	23.6	15.7	3.6	

In the Murine Melanoma—B16 mice model doses of 4000, 5000 and 6000 (mg/kg) were at least equal in tumor suppression compared to cytoxan at 30–60 days. Since this tumor is a liquid tumor, there is no change in tumor weight. The results are summarized in Table 1 below.

In the P-388 model for leukemia there was a dose responsive effect and the results were good. See Tables 2 and 3.

The data in Tables 1, 2 and 3 are reported in T/C which is interpreted using the following scale:

T/C <125	no activity
T/C = 125–150	weak activity
T/C = 150–200	modest activity
T/C = 200–300	high activity
T/C = 300 with long term survivors	excellent activity

long term survivors for P388 is >30 days, for B16 it is >60 days. The NCI Measure of Success is T/C=125.

Use of Carbendazim in the Treatment of B16

This study was performed in black mice injected i.p. with B-16 mouse melanoma which many researchers believe is the most predictive model for efficacy in humans. Carbendazim was equally effective as Cytosin in this model at treating B-16. The activity is dose responsive. Death is the end point in this model.

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TABLE 1

Dosage (mg/kg) twice weekly	T/C	weight change (%)	Positive Control
5000	198	-0.64	Cytosin at 300 mg/kg
2500	169	+3.16	one dose
2000	169	-11.63	T/C 191, -8.43%
1250	124	+1.99	weight change
1000	176	-4.75	
500	141	-0.64	

TABLE 2

In a repeat test the following results were achieved.			
Dosage (mg/kg) twice weekly	T/C	weight change (%)	Positive Control
6000	183	-0.38	Cytosin at 300 mg/kg
5000	167	-1.44	one dose
4000	138	+3.86	T/C 161, -4.28%
			weight change

TABLE 3

Use of Carbendazim in the treatment of P388			
Dosage (mg/kg)	T/C	weight change (%)	Positive Control
4000	189	-9	cytosin at 125 mg/kg
2000	148	+8	is curative; weight
1000	129	+15	change -14

Accordingly, carbendazim retards the growth of both solid and liquid tumors in vivo.

These studies confirm previous in vivo studies in which carbendazim was tested in various in vivo models for different types of cancer.

Additional in vivo cancer studies are presented in a tabular form below. The data is presented in the following format—dose regimen in parenthesis, dosage in mg/kg: tumor growth suppression (tg supp.), toxic deaths/number of test subjects (deaths), % weight change (wt).

TABLE 4

dose	tg supp.	deaths	wt.
Cancer			
MX1 xenograft - (twice weekly)			
6000	70%	1/9	-4
5000	63%	1/10	-4
4000	48%	0/10	-1
MCF-7L (breast)(twice weekly)			
6000		10/10	
5000	100%	7/10	-8
4000	94%	5/10	-7
DU-145 (twice weekly)			
6000	33%	1/10	-6
5000	48%	1/10	-8
4000	52%	0/10	-5
A549 (lung)			
2500		10/10	
500	57%	0/10	+10

TABLE 4-continued

dose	tg supp.	deaths	wt.
<u>HT29 (colon) (twice weekly)</u>			
6000	97%	9/10	-25
5000	79%	2/10	-8
4000	78%	3/10	-12
3000	65%	2/10	-6
2000	36%	0/10	+3
<u>SK-MES (twice weekly)</u>			
6000	69%	3/10	-2
5000	44%	1/10	+2
4000	45%	2/10	+3
<u>Positive Control</u>			
<u>Cytosin (1 dose)</u>			
300	complete shrinkage	0/10	-7
NCI Measure of Success: 58%			
<u>Cytosin (1 dose)</u>			
300	100%	3/10	-15
NCI Measure of Success: 58%			
<u>Cytosin (1 dose)</u>			
300	0%	0/10	+6
NCI Measure of Success: 58%			
<u>Cytosin</u>			
125	69%	0/10	-1
<u>Cytosin (1 dose)</u>			
300	45%	0/10	+10
NCI Measure of Success: 58%			
<u>Taxol (5 doses)</u>			
20	75%	0/10	+1
<u>Navelbine (5 doses)</u>			
2	26%	1/10	-5
NCI Measure of Success: 58%			

Carbendazim demonstrated the ability to reduce tumor growth in mice models for breast, lung, colon, murine melanoma and leukemia. The data are summarized in Table 5 and Table 6 below.

TABLE 5

Cancer	Dosage	Tumor Growth Decrease (%)	Positive Control (Dosage in mg/kg)	Tumor Growth Decrease
MXI-breast	500 mg/kg	42%	cytosin (125)	regression
	2500 mg/kg	37%		
A549 - lung	500 mg/kg*	57%	cytosin (125)	69%
HT29 - colon	2500 mg/kg**	54%	cis-plat (4)	59%

*in this model 2500 mg/kg was toxic;

**in this model 500 mg had no effect

In the same test 2-(4-thiazolyl)-1H-benzimidazole showed no activity against MXI breast cancer tumors implanted subcutaneously under the mice skin.

TABLE 6

Cancer	Dosage	Increased Life Span	Positive Control
5 P388-leukemia	1000 mg/kg	129%	cytosin (125) curative
	2000 mg/kg	148%	
	4000 mg/kg	decreased life span - 9%	
10 B16 melanoma	1000 mg/kg	131%	navelbine (2) increased life span - 265%
	2000 mg/kg	163%	
	4000 mg/kg	187%	

15 In the same test 2-(4-thiazolyl)-1H-benzimidazole showed no activity against P388.

In the same test 2-(4-thiazolyl)-1H-benzimidazole showed no activity against B16.

The in vivo and in vitro data support the assertion that carbendazim has broad scale efficacy against multiple cancer types.

20 Initial efficacy of carbendazim appears to be comparable to the best available drugs used for the treatment of any particular cancer type. Furthermore, with continuous treatment, breast cancers do not come back as usually happens with cytosin and taxol in breast cancer or gemcitabine in pancreatic cancer.

25 It is equally effective against p53 deficient/defective cell lines, unlike most existing cancer treatment drugs.

It is excellent in B16 mouse melanoma, which is believed by many people to be the best predictive model for efficacy in humans.

These same benzimidazole derivatives are effective against viruses including HIV, influenza, rhinoviruses and herpes viruses. The benzimidazole derivatives can be used alone or in combination with other fungicides.

35 The following examples illustrate the effectiveness of thiabendazole, 2-(4-thiazolyl)-1H-benzimidazole, against HIV and the benzimidazoles derivatives against a number of viruses.

The results of these HIV studies are summarized in more detail below:

Thiabendazole is effective at totally suppressing virus production in chronically infected cells. The extra cellular viral count goes effectively to zero or non-detectable levels. Thiabendazole does not kill the chronically infected cells though it does reduce the rate of cell proliferation at active concentrations. Thiabendazole does not affect CD₄ expression in uninfected cells. At effective concentrations thiabendazole slows but does not alter the normal cellular RNA or protein synthesis of either infected or non-infected cells.

Thiabendazole is effective in a variety of chronically infected cell types (this effect is not cell type specific.) Thiabendazole is effective against a variety of HIV virus strains. (Not virus strain specific—although some variance by strain is observed; SK-1>IIB>RF) Also thiabendazole is not effective on SIV in vitro or in vivo.

After 20 months no resistant virus strains to thiabendazole have developed in tests designed to do so. Resistance develops in six months or less in this test for existing HIV drugs with resistance strains for protease inhibitors developing in about 3-4 months.

Thiabendazole does not adversely affect the activity of existing HIV drugs, AZT, 3TC, ddC, ddI or protease inhibitors (saquinavir and indinavir) in acutely infected cells, nor do any of these existing drugs interfere with the efficacy of thiabendazole in chronically infected cells. It is used in combination with these drugs. Thiabendazole is also effective against protease inhibitor resistant viruses.

Thiabendazole confers temporary suppression of viral production from 4 to 80 days after treatment stops. This is unique and a useful feature whenever one has problems with compliance.

The results of these studies are summarized in detail below:

HIV Virus Replication Study

Thiabendazole was tested in chronically infected HIV virus. These cell populations contain integrated copies of the HIV genome and constitutively produce HIV at relatively high levels (CEM-SK1, U937-SK1 and H9-SK1 from Frederick Research Center, Md.) or are latently infected and only produce virus after stimulation with phorbol esters, tumor necrosis factor or IL6 (U1 and ACH2). Virus production was reduced in all cell lines tested and thiabendazole did not stimulate virus production from the latently infected cells. Reductions in virus production were observed when quantifying supernatant reverse transcriptase activity, supernatant p24 as well as intracellular p24, indicating the compound inhibits virus production at a step of replication prior to production of intracellular proteins.

Quantification of the infectivity of virions produced from the infected cells demonstrates reductions in the number of infectious virions in parallel with reductions in supernatant RT or p24, indicating the compound reduces the amount of virus produced, but not the quality of the virions. Inhibition of virus production from the chronically infected cells was observed at concentrations which were nontoxic to the target class. Thiabendazole inhibited virus production at concentrations greater than 1–10 $\mu\text{g/ml}$.

Toxicity to the chronically infected cells was similar to that observed with the uninfected cells. Evaluation of thiabendazole on chronically infected cells was performed by evaluation of thymidine (DNA), uridine (RNA) and leucine (protein) incorporation into cellular macromolecules. Inhibition of cellular macromolecule synthesis paralleled the toxicity of the compound as would be expected and did not occur at lower nontoxic concentrations found to inhibit virus production from the chronically infected cells.

After 28 days of treatment on chronically infected cells, the toxicity of the compound to the target cells appeared similar in both uninfected and chronically infected cells. The compound does not preferentially kill HIV-infected cells. Reductions in the level of virus production were stable and were observed at concentration greater than 10 $\mu\text{g/ml}$ for thiabendazole.

These results suggest thiabendazole can quickly reduce the level of virus production from cell populations chronically infected with HIV-1 and the antiviral effect is maintained with prolonged compound exposure. This reduction of virus production occurs at concentrations which are nontoxic to the host cell and which have no effect on the synthesis of cellular DNA, RNA and protein.

Virus Resistance Studies

Chronically infected HIV cells were cultured in the presence of thiabendazole at 1 $\mu\text{g/ml}$ for the first month, 5 μg for the second month, 10 $\mu\text{g/ml}$ for the third month, 20 and 40 $\mu\text{g/ml}$ for the fourth month and 80 $\mu\text{g/ml}$ for the fifth and sixth months. At the end of each month, the cells were evaluated for virus production compared to chronically infected cells not treated with the compound. For each of the six months of treatment experience, no change in the antiviral effect of the compound was noticed and the toxicity of the compound remains identical. Thiabendazole remains

active against HIV and that resistance was not rapidly achieved via the selection of resistant viruses or adaptation of the cells to prevent compound induced toxicity. Virus production remains totally suppressed from cultures treated with thiabendazole at 40 and 80 $\mu\text{g/ml}$.

The present invention includes a method of treatment HIV with the benzimidazole compounds without inducing formation of benzimidazole derivative or thiabendazole resistant HIV.

Reappearance of Virus Production from Chronically Infected Cells Previously Treated

Chronically infected cells which were treated with compound for prolonged periods were washed free of compound and cultured to determine it and when, virus production would resume. Cultures in which treatment resulted in the total elimination of virus production were used in these assays. These cultures included chronically infected cells cultured in the presence of 20, 40, and 80 $\mu\text{g/ml}$ of thiabendazole. Within 4 days virus production resumed from the cells cultured in the presence of the lower concentrations of thiabendazole (20 $\mu\text{g/ml}$ and 4 $\mu\text{g/ml}$). Virus production resumed at the 40 $\mu\text{g/ml}$ concentration of thiabendazole by day 12. At the highest concentrations virus production was observed at approximately day 70.

The present invention includes a method of treatment HIV with the benzimidazole derivative or thiabendazole and delaying the reappearance of HIV in plasma following initial treatment of HIV with an antiviral agent or thiabendazole.

Infectability of Cells Treated with Thiabendazole

Cells which were pretreated with thiabendazole for a long period of time were washed free of compound and used as a target cell population. The cells were split into 3 populations and labeled Group 1, 2 or 3. Group 1 was treated with the compound for 24 hours (at the same concentration used in the prolonged treatment phase), washed free of compound and cultured in the presence of infectious virus and fresh compound. Group 2 was pretreated for 24 hours, washed free of compound and cultured in the presence of infectious virus only. Group 3 was cultured for both the pretreatment and the infection phases in fresh medium only (no virus or compound). Virus production from the cell populations was identical irrespective of the culture conditions. These results indicate that the chronically infected cells treated for prolonged periods were not super-infected with HIV.

Additional Chronic HIV studies

Chronic HIV-1 infected cells U1 were derived from an acute HIV-1 infection of the promonocytic cell line, U937. The chronic HIV-1 infected cells, ACH-2 were derived from an acute HIV-1 infection of the T cell line, A3.01.

These cells were cultured in medium and the phorbol ester, PMA. PMA causes the cells (both U1 and ACH-2) to be activated and not divide but it also causes the U-1 cells to differentiate. This results in fewer cells in the PMA-treated cultures than the media alone cultures. Cell viability was measured when these cell lines were treated with the test compound.

Both cell lines constitutively produce a small amount of HIV-1. ACH-2 cell lines tend to produce more HIV-1 than U1 cells as shown by p-24 ELISA. When either cell line is cultured in the presence of PMA there is an increase in the quantity of HIV-1 produced as measured by the p-24 antigen ELISA.

In addition, the number of institute positive HIV mRNA expressing cells per microscopic field is measured. Comparisons can be made from these numbers since the same number of cells were adhered to the glass slides for each drug concentration (10×10^6 cells/ml).

These cells were treated with test samples. Thiabendazole at 60 $\mu\text{g/ml}$ suppressed replication in the HIV monocytes by 74% and the T-cell HIV replication was increased by 26%. The positive control was interferon which suppressed HIV monocytes replication by 80%. AZT showed no activity in this model.

2-(Methoxycarbonylamino)benzimidazole suppressed replication in the HIV monocytes by 9% and the T-cell HIV replication was increased by 44%. The positive control was interferon which suppressed HIV monocytes replication by 80% and suppressed T-cell HIV replication by 60%.

Acute HIV Testing

In an in vitro acute model for HIV 2-(methoxycarbonylamino)benzimidazole inhibited viral replication by 100% at 4 $\mu\text{g/ml}$ and AZT inhibited viral replication by 98% at 1 $\mu\text{g/ml}$. 2-(4-thiazolyl)-1H-benzimidazole inhibited viral replication by 98% at 60 $\mu\text{g/ml}$.

The therapeutic index (TI), the ratio of the toxic dose of drug to efficacious dose of drug for 2-(4-thiazolyl)-1H-benzimidazole is 2.8 versus 12,500 for AZT. The TI for 2-(methoxycarbonylamino)benzimidazole is 1.8.

In Vivo Herpes

In an in vivo herpes screening test of 2-(4-thiazolyl)-1H-benzimidazole at a dose of 200 mg/kg dose, 10% of the mice survived with a 10.4 mean death date; at 100 mg/kg dose 50% of the mice survived with a 9.2 mean death date. The positive control was acyclovir at 75 mg/kg dose; 60% of the mice survived with a mean death date of 17.2 days. In the same test 2-(methoxycarbonylamino)benzimidazole showed no activity.

Other Tests

Both 2-(4-thiazolyl)-1H-benzimidazole and 2-(methoxycarbonylamino)benzimidazole were tested in an in vitro influenza model and showed no activity.

In an in vivo model for influenza 2-(4-thiazolyl)-1H-benzimidazole was tested at 200 mg/kg, 67% of the mice survived with a mean death date of 8 days; at 100 mg/kg, 62% survived with a mean death date of 8.7 days. The positive control was amantadine (75 mg/kg) with 100% of the mice surviving for 21 days. 2-(Methoxycarbonylamino)benzimidazole was not active in the same test.

Both 2-(4-thiazolyl)-1H-benzimidazole and 2-(methoxycarbonylamino)benzimidazole were tested in an in vitro herpes model and showed no activity.

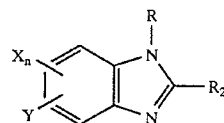
Both 2-(4-thiazolyl)-1H-benzimidazole and 2-(methoxycarbonylamino)benzimidazole were tested in an in vitro rhinovirus model and compared to A-36683. The therapeutic index (TI), the ratio of the toxic dose of drug to efficacious dose of drug, for 2-(4-thiazolyl)-1H-benzimidazole is 1-2 and for 2-(methoxycarbonylamino)benzimidazole is 1-3 versus 1000-3200 for A-36683.

The demonstrated effectiveness of the compounds of the present invention in the human breast and lung tumor xenograft models indicate that the compounds of the present invention are useful for the treatment of solid tumors in man, and, in particular, tumors of the breast and lung. This

conclusion is further supported by published analyses correlating pre-clinical test results with clinical efficacy of anti-cancer agents. For example, see: Goldin and Venditti (1980) Recent Results Cancer Research 76: 176-191; Goldin et al. (1981) Eur. J. Cancer 17: 129-142; Mattern et al. (1988) Cancer and Metastasis Review 7: 263-284; Jackson et al. (1990) Cancer Investigations 8: 39-47. Based on these published analyses, the exceptional high level of antitumor activity exhibited by the presently claimed compounds provide strong evidence that the compounds claimed in present invention have therapeutic utility in the treatment of cancer in man and that they will improve the quality of life of the patient.

What is claimed is:

1. A method of treating cancer selected from the group consisting of carcinoma, sarcoma, and lymphoma, the method comprising administering to a patient in need thereof a therapeutically effective amount of a composition comprising a benzimidazole compound of the formula:



wherein,

X is hydrogen, halogen, alkyl of less than 7 carbon atoms, or alkoxy of less than 7 carbon atoms;

n is a positive integer of less than 4;

Y is hydrogen, chloro, nitro, methyl, ethyl, or oxychloro;

R is hydrogen, an alkyl group of from 1 to 8 carbon atoms, or alkylaminocarbonyl wherein the alkyl group has from 3 to 6 carbon atoms; and

R₂ is NHCOOR₁, wherein R₁ is an aliphatic hydrocarbon of less than 7 carbon atoms;

or a prodrug thereof, a pharmaceutically acceptable salt thereof, or mixtures thereof.

2. A method according to claim 1 wherein said cancer is prostate cancer.

3. A method according to claim 1 wherein said cancer is melanoma.

4. A method according to claim 1 wherein said cancer is Rhabdomyosarcoma.

5. A method according to claim 1 wherein said cancer is pancreatic cancer.

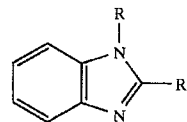
6. A method according to claim 1 wherein said cancer is neuroblastoma.

7. A method according to claim 1 wherein said cancer is cervical cancer.

8. A method according to claim 1 wherein said cancer is ovarian cancer.

9. A method according to claim 1 wherein said cancer is stomach cancer.

10. A method according to claim 1 wherein said benzimidazole compound is of the formula:



wherein R is an alkyl of 1 to 8 carbon atoms, and R₂ is NHCOOR₁ wherein R₁ is methyl, ethyl or isopropyl; or a

non-toxic, pharmaceutically acceptable acid salt of said benzimidazole compound; or a prodrug of said benzimidazole compound; or mixtures thereof.

11. A method of treating cancer according to claim 1, wherein recurrence of the cancer is inhibited by the benzimidazole compound.

12. A method of treating cancer according to claim 1, wherein the benzimidazole compound induces apoptosis in cancer cells at sub-lethal concentrations to normal cells.

13. A method of treating cancer according to claim 1, wherein the cancer has survived treatment with another anticancer agent prior to treatment with the benzimidazole compound.

14. A method of treating cancer according to claim 1, wherein the growth rate of the cancer is reduced by the benzimidazole during exposure of the cancer to estrogen.

15. A method according to claim 1 wherein said pharmaceutically acceptable salt is hydrochloride salt.

16. A method according to claim 1 wherein said benzimidazole compound is administered in an amount of from 30 mg/kg of body weight to 10,000 mg/kg of body weight.

17. A method according to claim 1 wherein said benzimidazole compound is administered in the form of a liposome delivery system.

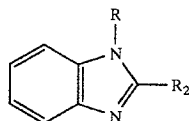
18. A method according to claim 1 wherein said benzimidazole compound is in the form of a parenteral composition suitable for administration by injection.

19. A method according to claim 1 wherein said cancer is lung cancer.

20. A method according to claim 1 wherein said cancer is breast cancer.

21. A method according to claim 1 wherein said cancer is colon cancer.

22. A method according to claim 1 wherein said benzimidazole compound is of the formula:



wherein R is hydrogen or alkylaminocarbonyl wherein the alkyl group has from 3 to 6 carbon atoms, and R₂ is NR₁COOR₁, wherein R₁ is methyl, ethyl or isopropyl; or a non-toxic, pharmaceutically acceptable salt of said benzimidazole compound; or a prodrug of said benzimidazole compound; or mixtures thereof.

23. A method of treating cancer selected from the group consisting of carcinoma, sarcoma, and lymphoma, the

method comprising administering to a patient in need thereof a therapeutically effective amount of a composition comprising 2-(methoxycarbonylamino)benzimidazole.

24. A method of treating cancer selected from the group consisting of carcinoma, sarcoma, and lymphoma, the method comprising administering to a patient in need thereof a therapeutically effective amount of a composition comprising methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate.

25. A method according to claim 23 wherein said 2-(methoxycarbonylamino)benzimidazole is in the form of a prodrug thereof.

26. A method according to claim 23 wherein said 2-(methoxycarbonylamino)benzimidazole is in the form of a pharmaceutically acceptable salt thereof.

27. A method according to claim 26 wherein said pharmaceutically acceptable salt is hydrochloride salt.

28. A method according to claim 23 wherein said 2-(methoxycarbonylamino)benzimidazole is administered in an amount of from 30 mg/kg of body weight to 10,000 mg/kg of body weight.

29. A method according to claim 23 wherein said 2-(methoxycarbonylamino)benzimidazole is administered in the form of a liposome delivery system.

30. A method according to claim 23 wherein said 2-(methoxycarbonylamino)benzimidazole is in the form of a parenteral composition suitable for administration by injection.

31. A method according to claim 24 wherein said methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate is in the form of a prodrug thereof.

32. A method according to claim 24 wherein said methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate is in the form of a pharmaceutically acceptable salt thereof.

33. A method according to claim 32 wherein said pharmaceutically acceptable salt is hydrochloride salt.

34. A method according to claim 24 wherein said methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate is administered in an amount of from 30 mg/kg of body weight to 10,000 mg/kg of body weight.

35. A method according to claim 24 wherein said methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate is administered in the form of a liposome delivery system.

36. A method according to claim 24 wherein said methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate is in the form of a parenteral composition suitable for administration by injection.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,262,093 B1
DATED : July 17, 2001
INVENTOR(S) : James Berger Camden

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:


Column 29,

Line 44, delete "NRCOOR₁" and insert in lieu thereof -- NHCOOR₁ --.

Signed and Sealed this

Fourteenth Day of May, 2002

Attest:

A handwritten signature in black ink, appearing to read "James E. Rogan", with a horizontal line drawn underneath it.

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

EXHIBIT 2

ORIGINAL PAPER

Jose Antonio Perdomo · Yoshio Naomoto
Minoru Haisa · Toshiyoshi Fujiwara · Madoka Hamada
Yasuo Yasuoka · Noriaki Tanaka

In vivo influence of p53 status on proliferation and chemoradiosensitivity in non-small-cell lung cancer

Received: 14 August 1997 / Accepted: 10 October 1997

Abstract Alteration of the *p53* gene product is a frequent event in the progression of lung cancer. However, its importance to proliferation and response to chemoradiotherapy remains unclear. Thus, to assess its influence directly in vivo, we implanted into nude mice two kinds of human non-small-cell lung cancer (NSCLC) cells: H226br having a homozygous gene mutation in *p53* (mt-*p53*) and H226b with intact *p53* (wt-*p53*). We found that mt-*p53* tumors grew substantially faster than wt-*p53* tumors. Furthermore, treatment with cisplatin and radiation did not reduce the size of mt-*p53* tumors, while wt-*p53* tumors regressed by approximately 60%. Terminal-deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling assay revealed apoptosis to be the mechanism responsible for the regression. Interestingly, apoptosis occurred in mt-*p53* tumors although only at high doses of cisplatin and not at the magnitude detected in wt-*p53* tumors. Cell labeling by staining with bromodeoxyuridine indicated that *p53* is an important factor in modulating growth in NSCLC tumors. Our results are consistent with the notion that correction of a single genetic lesion enhances the therapeutic effect of chemotherapy.

Key words *p53* · Chemotherapy · Radiation · Apoptosis · Proliferation

Abbreviations NSCLC non-small-cell lung cancer · wt wild type · mt mutant type · TUNEL terminal-deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling

Introduction

The tumor suppressor *p53* is a widely studied transcription factor and its inactivation by sense mutation or deletion, the commonest genetic alterations in human cancer (Levine et al. 1991; Hollstein et al. 1991), occurring in as many as 80% of cases in some cancers (Greenblatt et al. 1994), plays an important role in the pathogenesis of neoplasia.

An altered *p53* gene abolishes the tumor-suppressor activity of the *p53* protein, facilitating tumor development and increase of metastatic potential and tissue invasiveness (Hsiao et al. 1994), as has been observed in many types of human cancers, including brain (Sidransky et al. 1992), breast (Peyrat et al. 1995) and colon cancer (Kastrinakis et al. 1995).

p53 protein has also been shown to play an important role in the response to DNA damage induced by chemotherapeutic drugs and/or ionizing radiation (Tishler et al. 1993). The mechanism involved entails a rapid increase in *p53* protein levels and the mediation of several cellular responses, including (1) G1 arrest via transcriptional induction of p21/WAF21 (El Deiry et al. 1994; Chen et al. 1995), (2) DNA damage repair via transcriptional induction of GADD45 (Kastan et al. 1992), which interacts with PCNA to carry out this function (Smith et al. 1994), and (3) induction of apoptosis (Lowe et al. 1993a, b).

It has been shown that the arrest of the cell cycle at G1 allows cells to repair DNA before S-phase. If optimal repair is impossible, *p53* then triggers programmed cell death (Smith et al. 1994; Fritsche et al. 1993). In tumoral cells with mutant-type *p53* (mt-*p53*), the loss of *p53* function, and subsequently the above cellular events, is thought to result in resistance to chemotherapeutic agents (Lowe et al. 1993b; O'Connor et al. 1993; Lee and Bernstein 1992; Fan et al. 1994). However, in some cells, the loss of the G1 checkpoint does not influence chemoradiosensitivity, since the G2 checkpoint may be a more important determinant of sensitivity

J. A. Perdomo (✉) · Y. Naomoto · M. Haisa
T. Fujiwara · M. Hamada · Y. Yasuoka · N. Tanaka
First Department of Surgery,
Okayama University Medical School, Shikata-cho, 2-5-1,
Okayama 700, Japan
Tel.: +86 235 7260; Fax: +86 221 8775

(Slichmyer et al. 1993). The differences described probably indicate that the kind of response is a cell-type-specific phenomenon.

Recently, studies performed *in vitro* have provided new insights into the cellular mechanism involved in the induction of apoptosis by chemotherapeutic agents. However, relatively little is known about the relationship between p53 status, apoptosis and DNA damage *in vivo*. Cisplatin, a DNA-cross-linking chemotherapeutic drug, and radiation, a treatment that produces single-strand breaks in DNA, are widely used to treat locally advanced non-small-cell lung cancer (NSCLC). Unfortunately, few patients develop a complete pathological response. The factors responsible for this lack of response are multiple and not well understood (Reeve et al. 1996; Tsai et al. 1996). Thus, to elucidate the relation of p53 status to proliferation and response of NSCLC tumors to these agents *in vivo*, we selected and implanted into nude mice two human non-small-cell lung cancer lines differing only in p53 status.

Materials and methods

Cell lines

The human squamous NSCLC cell line H226br, with a homozygous mutation in p53 (mt-p53) at codon 254, and H226b, having an intact p53 gene [wild type (wt-p53)], were used. H226br is a variant of the H226b cell line derived from a brain metastasis in a nu/nu mouse (gift of I.J. Fidler, the University of Texas M.D. Anderson Cancer, Houston) (Cai et al. 1995). The H226b cell line was a gift from A. Gazdar and J. Minna (Simmons Cancer Center, Dallas, Texas) and have been previously described (Mitsudomi et al. 1992; Fujiwara et al. 1994). These tumor cells were maintained in RPMI-1640 medium (Sanko Junyaku Co. Ltd., Tokyo) containing 10% fetal bovine serum.

Animals

Male athymic specific-pathogen-free nude mice of a CD-1 genetic background and weighing 20–25 g were obtained from Charles River Japan and used at 4 weeks of age. The mice were kept in sterilized cages equipped with an air filter and sterile bedding material, and fed with sterilized water and food *ad libitum*.

Tumoral cell transplantation and evaluation of antitumor effects

H226b and H226br cells (4×10^6 cells/0.2 ml cell suspension) were subcutaneously injected into the back of each mouse. The tumors were measured by length and width, using calipers, three or four times weekly by the same observer. Tumor weight was calculated from linear measurements according to the Battelle Columbus protocol: tumor weight (mg) = $1/2 \times a \times b^2$, where a is the length (mm) and b the width (mm).

At around 4 weeks after the inoculation of tumoral cells, when the tumors had reached a weight of around 90–120 mg, tumor-bearing mice were randomly allocated to groups with at least five mice in each group and irradiated or intraperitoneally treated with cisplatin. The antitumor response was evaluated using one dosification and expressed as relative weight, using the following formula: relative tumor weight = mean tumor weight at a given time/initial mean tumor weight.

Apoptotic response to chemo- and radiotherapy

The tumors were allowed to grow for 4 weeks until they weighed about 90–120 mg. Cisplatin (Briplatin; Bristol-Myers Squibb K.K., Tokyo, Japan) was used. The drug at 3 mg/kg and 15 mg/kg was administered *i.p.* using a 26-gauge tuberculin needle. Mice were sacrificed 24 h, 48 h and 72 h after treatment and the tumors were resected, fixed in formalin and embedded in paraffin to be processed for histological examination by nick-end labeling.

Different sets of five tumor-bearing mice were irradiated once with 4 Gy and 12 Gy from a Hitachi Medico (MBR 1520) X-ray generator delivering a dose rate of 1.5 Gy/min. After 24 h, 48 h and 72 h, mice were sacrificed and tumors were resected for histological evaluation.

Detection of apoptotic cells by *in situ* DNA nick-end labeling

As the reliability has been confirmed (Veis et al. 1993), we employed the method described by Gavrieli et al. (1992) for the identification of apoptotic nuclei. In brief, paraffin sections of formalin-fixed tissues were dewaxed, rinsed in alcohol and allowed to dry. Sections were then digested with proteinase K (20 µg/ml) for 15 min at 37°C and the slides washed four times in double-distilled water for 2 min. Endogenous peroxidase was inactivated by covering the sections with 2% H₂O₂ for 5 min at room temperature. The sections were rinsed with double-distilled water and immersed in TDT buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). TDT and biotinylated dUTP in TDT buffer were added to cover the sections, which were then incubated in a humid atmosphere at 37°C for 60 min. The reaction was terminated by transferring the slides to buffer containing 300 mM sodium chloride/30 mM sodium citrate for 15 min at room temperature. The sections were rinsed with double-distilled water, covered with a 2% aqueous solution of human serum albumin for 10 min at room temperature, rinsed in double-distilled water and immersed in phosphate-buffered saline (PBS) for 5 min. The sections were treated with peroxidase-conjugated streptavidin (Vectastain Elite ABC Kit) for 30 min at 37°C, washed in double-distilled water, immersed for 5 min in PBS, stained with diaminobenzidine/H₂O₂ solution (DAB substrate kit, Histofine, Tokyo, Japan) for about 10 min at room temperature and counter-stained with methyl green overnight.

An apoptotic labeling index was calculated after 5000 nuclei in randomly selected fields had been counted for each tumor under a light microscope using $\times 40$ objective and $\times 10$ ocular lenses.

Apoptotic response to chemo- and radiotherapy *in vitro*

H226b and H226br cells were seeded in RPMI-1640 medium at 37°C in a 5% CO₂ atmosphere in tissue-culture flasks at 4×10^5 cells/flask. On the second day, the medium was exchanged and cisplatin at different concentrations was added. Additional cultures without the drug were used as controls. At different times, the cells were collected, washed with PBS and smeared by cytopsin (1000 rpm for 5 min) on glass slides pretreated with neoprene/toluene solution. The slides were fixed in cold acetone for 10 min and then air-dried. Slides were then rinsed with PBS, covered with 0.02% Nonidet P-40 for 4 min and washed in PBS for 1 min, four times. Slides were subsequently treated as for the tissue preparations except that the incubation with proteinase K was omitted (Gavrieli et al. 1992).

Another set with the same cell concentrations and culture conditions was prepared. On the second day of culture, cells were irradiated using a Hitachi, Medico (MBR 1520R) X-ray generator at a rate of 1.5 Gy/min at various doses. At different times, the cells were collected and prepared for nick-end labeling as above.

Identification of bromodeoxyuridine(BrdU)-labeled cells

Mice were sacrificed 90 min after intraperitoneal (*i.p.*) injection of 10 mg/kg BrdU. Tumors were resected, fixed in formalin, and

embedded in paraffin. For immunohistochemical staining, the sections were deparaffinized, washed with distilled water for 5 min, and incubated with 0.3% H_2O_2 in methanol for 30 min to inactivate endogenous peroxidase. Washed with PBS, these sections were boiled in citrate buffer solution (pH 6.0) in an autoclave at 120°C for 15 min. After washing in PBS for 20 min, the sections were incubated in normal horse serum for 20 min at room temperature. They were then incubated with primary antibody for 12 h at 4°C in a moisture chamber. A Vectastain Elite kit (Vector Laboratories, Burlingame, Calif., USA) was used to apply the biotinylated secondary antibody (incubated for 30 min at room temperature), then avidin-biotin complex conjugated to horseradish peroxidase was applied (incubation for 30 min at room temperature). After washing in PBS for 10 min, these sections were incubated in diaminobenzidine/ H_2O_2 solution (DAB substrate Kit, Histofine, Tokyo, Japan) for 10–15 min at room temperature. As a negative control, a primary antibody was used for mouse IgG. For counterstaining, sections were incubated in methyl green solution. As a primary antibody, mouse monoclonal antibody Bu20a (Magaud et al. 1989) (diluted 1:20) was used.

Flow cytometry

H226b and H226br cells (1×10^6) were treated with cisplatin and radiation. After being suspended in trypsin, cells were washed in PBS, fixed in 70% ethanol and stored at 4°C. Cells were then washed with PBS, treated with RNase (500 units/ml) and Triton X-100 and finally stained with propidium iodide at 100 μ g/ml in PBS. Cell-cycle analysis was performed on a flow cytometer FACS Calibur (Becton Dickinson) and Cell Quest software provided by the manufacturer. A sample of 20000 cells was counted for each determination.

Determination of p53 status

Western blot analysis

After trypsinization, cells were washed once with ice-cold PBS, then solubilized in sodium dodecyl sulfate (SDS) sample buffer (125 mM TRIS pH 6.8, 4% SDS, 20% glycerol). Lysates were sonicated on ice and centrifuged at $15800 \times g$ for 15 min. The supernatant was stored at -70°C. Protein concentrations were determined by DC protein assay (Bio-Rad). Total cell lysates were run on SDS/polyacrylamide gels and transferred to nitrocellulose membranes. Western blots were done using a 1:1000 dilution of anti-p53, followed by a 1:10000 dilution of secondary anti-mouse IgG conjugated to horseradish peroxidase. Bands were visualized on autoradiography film with enhanced chemoluminescence detection reagents (Amersham Life Science). For a primary antibody, we used mouse monoclonal antibody Pab 1801.

Immunocytochemical staining for identifying p53 protein accumulation

Cytospin preparations were fixed in ethanol for 10 min. Subsequent steps were done as described in BrdU labeling. Primary antibody Pab 1801 (diluted 1:500) was used.

Statistical analysis

Data were analyzed by the Mann-Whitney test.

Results

The p53 status of the cell lines used was unchanged, as confirmed by Western blotting and immunocytochemical methods (Figs. 1, 2).

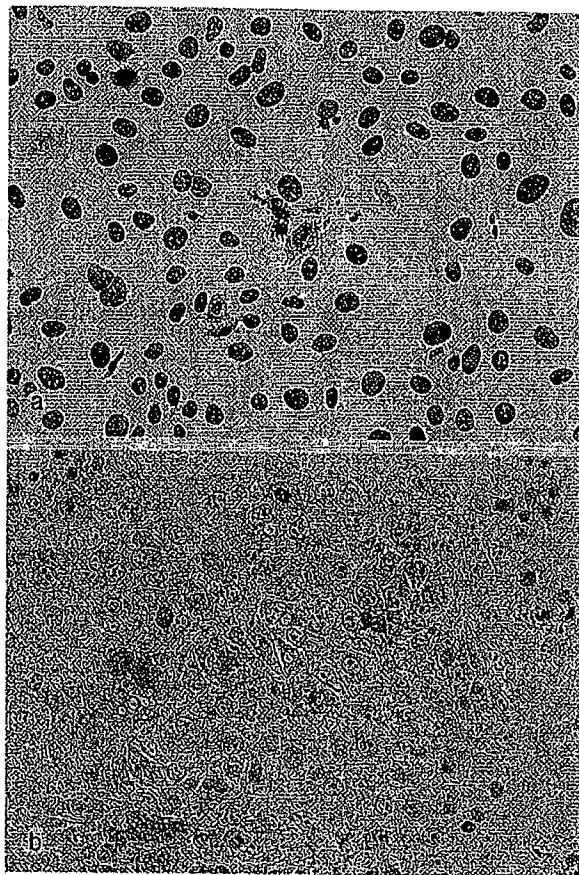


Fig. 1A, B Immunocytochemical staining for p53 protein. A p53-positive staining in non-small-cell lung cancer (NSCLC) cell line H226br. B p53-negative staining in NSCLC cell line H226b

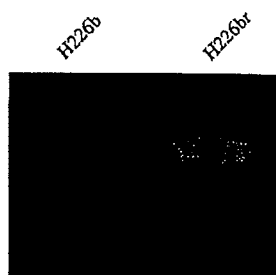


Fig. 2 Western blot of NSCLC cell lines H226b and H226br

Tumor growth

Growth rates of tumors derived from H226b and H226br cells were strongly influenced by the presence or absence of activity of the p53 gene product (Fig. 3). Until around day 40, the growth rate of both types of tumors was similar, with the mt-p53 tumors somewhat predominating. Thereafter, however, the growth of tumors with mutant p53 accelerated significantly.

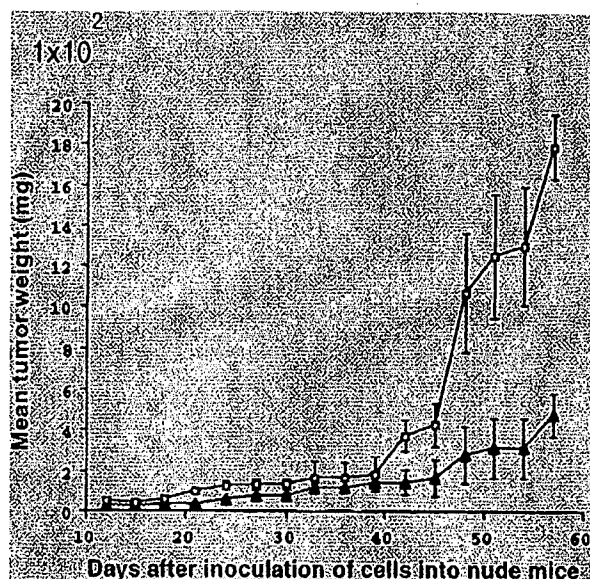
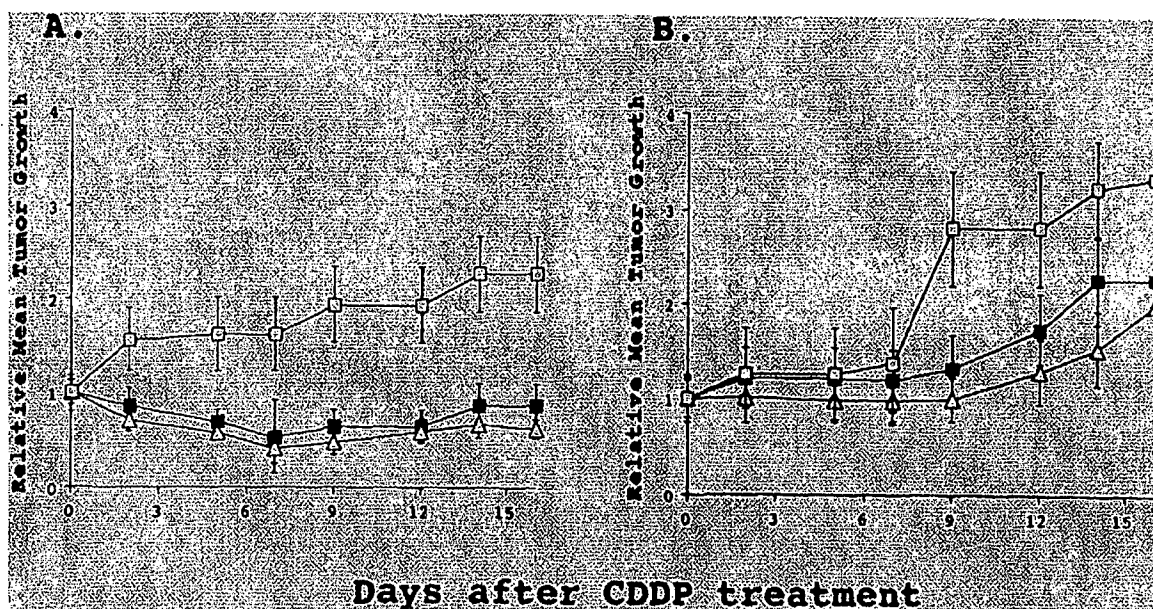


Fig. 3 Growth of tumors derived from H226b (p53 wild type) and H226br (p53 mutant type) cells inoculated into nude mice. No treatment. Note the only differences between these cell lines is p53 status. Each point shows the mean \pm SE of five tumors in one of three experiments. \blacktriangle H226b, \square H226br

Fig. 4A, B. Effect of cisplatin (CDDP) on the in vivo growth of tumors. Cisplatin at different concentrations was administered to mice bearing p53 wild- (A) and mutant-type (B) tumors. Tumor growth after treatment was monitored by measuring relative tumor size. Each point shows the mean \pm SE of five tumors in one of three experiments. \square Control, \blacksquare 3 mg, \triangle 15 mg



Inhibition of tumor growth by cisplatin and radiation

The effect of cisplatin administration is shown in Fig. 4. By day 2 after the administration of 3 mg/kg and 15 mg/kg cisplatin, the wt-p53 tumors (Fig. 4A) had already shown a regression of about 14% and 29% respectively. This response continued until day 7, when the regression was maximum (60%) and then growth slowly re-started. At all assay times, the regression was accentuated in the tumors treated with high doses.

In tumors of mt-type p53 (Fig. 4B), the main effect was a delay in growth, which lasted for around 1 week and, again, the more marked response was observed in tumors treated with high doses. No regression was observed.

Radiation of wt-p53 tumors at 4 Gy and 12 Gy led to a reduction in tumor size to around 78% and 55% of the pre-treatment size by days 2 and 4 respectively, but this response was of short duration and, around day 3, growth re-started. mt-p53 tumors showed a similar response although slower (Fig. 5).

Apoptosis induced by chemoradiation

Cells with features of apoptosis were detected by the terminal-deoxynucleotidyl transferase-mediated deoxyuridine-triphosphate-biotin nick-end labeling method (Fig. 6). The apoptotic response to cisplatin is shown in Fig. 7. Apoptosis was induced in both tumor types although to what extent related directly to p53 status. Within 24 h after administration of cisplatin, the percentage of apoptotic cells was higher in wild-type than mutated tumors ($P < 0.001$). This response persisted for 48 h and 72 h after treatment. In mt-p53 tumors, the highest re-

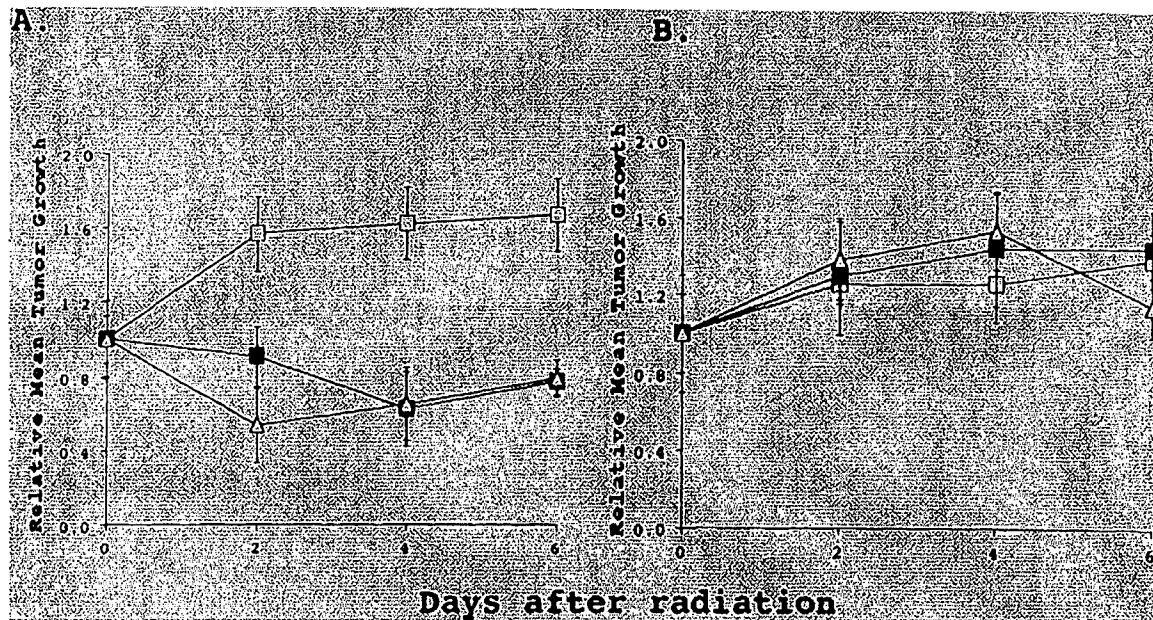
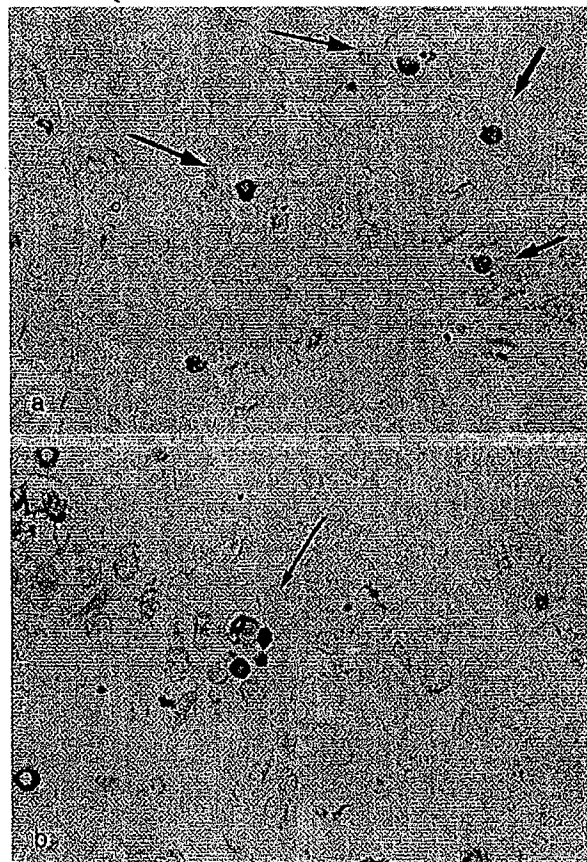


Fig. 5A, B Effect of radiation on the in vivo growth of tumors derived from p53 wild- (A) and mutant-type (B) cells. Each point shows the mean \pm SE of five tumors in one of three experiments. □ Control, ■ 4 Gy, △ 12 Gy



sponse was seen 48 h after treatment, but only after administration of a high dose cisplatin, and it never reached the level observed in wt-p53 tumors. A degree of apoptosis was also observed in untreated tumors.

Neither tumor type showed a sustained increase in the percentage of apoptotic cells after radiation, by nick-end labeling. Only 6 h after irradiation did the number of apoptotic cells increase in the wt-p53 tumors to around 2% of the total cells, and this subsequently declined sharply to pretreatment values without further increase (data not shown).

Labeling with BrdU

Marked differences between the two tumor types were observed after treatment with cisplatin (Fig. 8). In the wild-type tumors, a slight decrease in the number of labeled cells was observed (no statistical difference was found) although this decrease was accentuated in tumors treated at high doses. The opposite effect was induced in tumors of mt-p53. The response was maximized at low dose 72 h after treatment and at high dose 48 h after. Thereafter, the number of labeled cells decreased.

BrdU is a pyrimidine analog incorporated into cells during the DNA synthesis phase (S-phase). Therefore,

Fig. 6A, B Photomicrographs of a tumor derived from H226b cells after treatment with cisplatin. Scattered cells (A) with nuclear condensation, fragmentation and formation of apoptotic bodies (B) detected by terminal-deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling. Original magnification $\times 400$

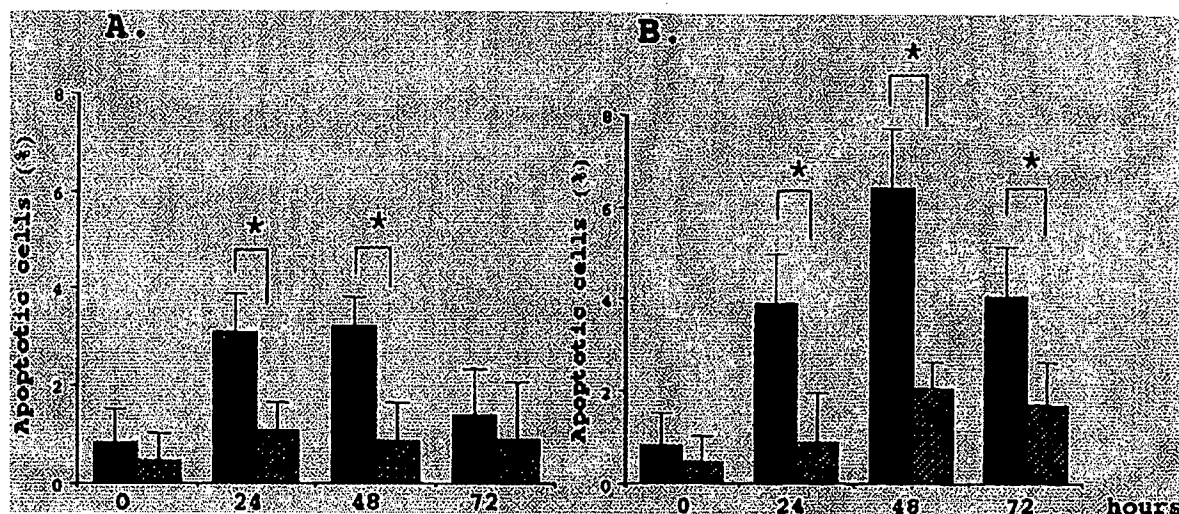


Fig. 7A, B. Apoptosis induced by administration of cisplatin at 3 mg/kg (A) and 15 mg/kg (B) in mice bearing wild-type p53 (black bars) and mutant-p53 (shaded bars) tumors. Data represent the mean \pm SE * P < 0.001

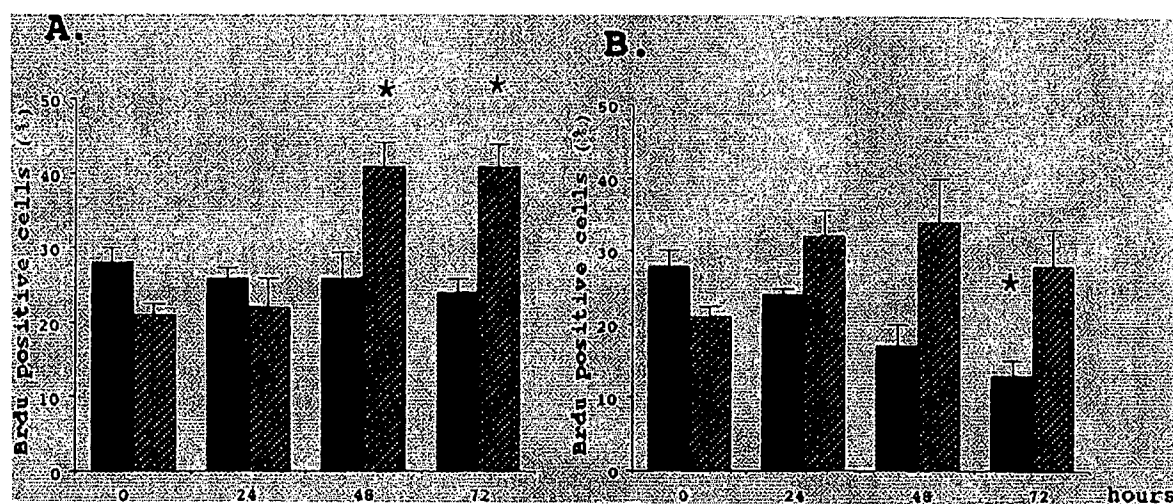


Fig. 8A, B Effect of administration of cisplatin at 3 mg/kg (A) and 15 mg/kg (B) on BrdU-labeled cells (S-phase cells) of tumors with different p53 status. Black bars wild type p53 tumors, shaded bars mutant p53 tumors, Data represent the mean \pm SE * P < 0.001 against control

the percentage of BrdU-positive cells represents the proportion of cells in the S-phase (Quinn and Wright 1992).

Thus in the first hours after the administration of cisplatin, the presence of wild-type p53 correlated with a decrease in the number of cells in S-phase. However, when p53 function was lost, tumoral cells in the S-phase accumulated.

In irradiated tumors, the response was similar for both tumor types, though more marked in wt-p53 tumors. The response varied in mt-p53 tumors (data not shown).

Apoptosis induced by chemoradiation in vitro

In vitro, exposure to cisplatin induced apoptosis in both tumor types (Fig. 9); however, the grade of response was

quite different. While, in wt-p53 cells, a concentration of 0.5 μ g/ml cisplatin was enough to induce a significant increase in the number of apoptotic cells, 10 μ g/ml was needed for mt-p53. The magnitude of response also differed at lower doses of cisplatin.

An apoptotic response occurred in irradiated cells, but it did not correlate with dose and/or p53 status (data not shown).

Cell-cycle analysis using flow cytometry

Fluorescence-activated cell sorting analysis 24 h after treatment of wt-p53 cells with cisplatin revealed an increase in G0/G1 cells with a simultaneous decrease of cells in S-phase, an effect enhanced at 1.0 μ g/ml (Table 1). mt-p53 cells showed no change in relation to G0/

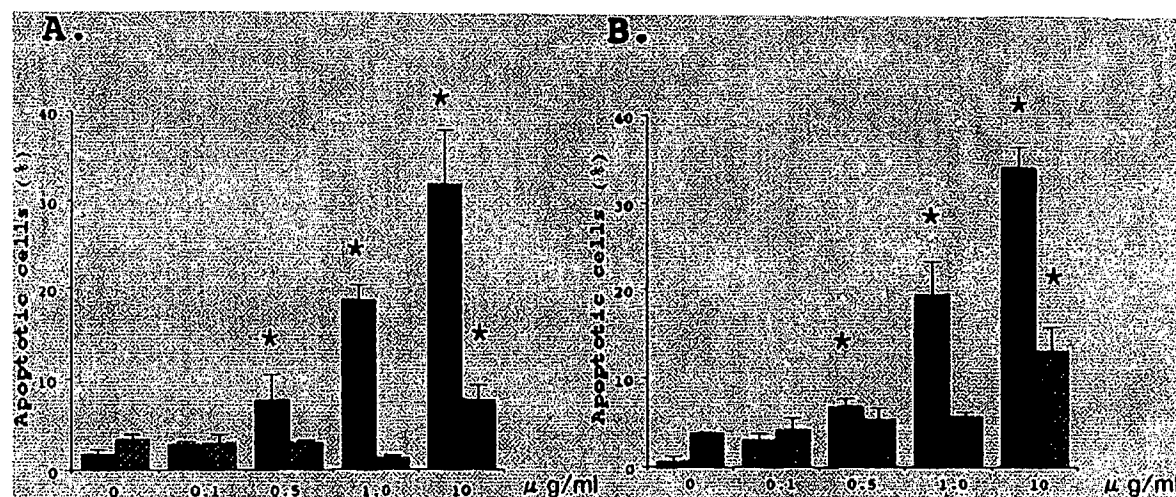


Fig. 9A, B Apoptosis induced 24 h (A) and 48 h (B) after administration of cisplatin at different concentrations in vitro. Black bars wild-type p53 cells, shaded bars, mutant p53 cells. Data represent the mean \pm SE * P < 0.001 against control

Table 1 Cell-cycle analysis 24 h after administration of cisplatin in H226b and H226r cells

Cell type	Cisplatin dose (µg/ml)	Cells in each phase (%)		
		G0/G1	S	G2M
H226b	Control	60	38	2
	0.5	50	41	9
	1.0	70	28	2
	10	66	24	10
H226br	Control	81	9	10
	0.5	80	20	0
	1.0	76	24	0
	10	78	15	7

G1 cells; however, S-phase cell numbers rose after 24 h. At 10 µg/ml, the number of S-phase cells returned to pretreatment values. No G2M arrest was evident.

When wt-p53 cells were irradiated, the number of G0/G1 cells had increased at 24 h. However there was no change in S-phase cell numbers at that time. In mt-p53 cells, a decrease in the number of G0/G1 cells accompanied an increase in cells in S-phase (data not shown).

Discussion

In cancer, the p53 gene product, a nuclear phosphoprotein, has been related to proliferation and response to DNA damage by chemotherapeutic agents. In lung cancer, alteration of p53 occurs in around 60% of cases (Chiba et al. 1990) and has been proven to be of prognostic significance especially in NSCLC (Fujino et al. 1995).

In this study, we sought to determine in vivo (1) the importance of this factor in the growth of lung tumors; (2) the relationship between p53 status and the effect of

chemoradiotherapy, (3) the importance and effect of p53-dependent and -independent apoptosis in the response to chemoradiation and (4) the influence of p53 status on the response of the cell cycle to DNA-damaging agents.

One of the main functions of p53 is suppression of cell proliferation, although the precise mechanism of this suppression is unknown (Allred et al. 1993). Both in vitro and in vivo, this function has been re-established after the introduction of a p53 gene with a virus vector (Fujimura et al. 1994; Liu et al. 1994). However, loss of function is not the only factor responsible for the increased tumorigenesis seen in tumors without p53. It has been reported that transfection of mt-p53 in human osteosarcoma and T cell acute lymphoblastic leukemia produces a gain of function facilitating tumor development and metastatic potential (Dittmer et al. 1993; Hsiao et al. 1994).

To test in vivo the importance of this factor in the development of tumors in lung cancer, we selected two human NSCLC lines with similar genetic characteristics, differing only in p53 status. We found that, while the growth of wt-p53 tumors was relatively slow, that of mt-p53 tumors was uncontrollable. This would not be surprising considering that the mutation of the cells used in this experiment is located between codons 100 and 295 (specifically codon 254), which is considered a critical region essential for growth suppression (Srinivasan et al. 1993). Therefore, the loss of activity of the p53 gene could be responsible for the accelerated growth observed in the mt-p53 tumors. Considering that around 60% of NSCLC tumors present some kind of mutation in the p53 gene, this fact could explain, at least in part, their poor prognosis, especially in patients in whom the tumor is not surgically resectable because of extensive local invasion.

However, the potential prognostic importance of this factor in NSCLC is not limited. A recent report has

linked anomalous p53 expression with resistance to chemotherapy (Rusch et al. 1995). In fact, the chemotherapeutic regimens are based on cisplatin. It has been described how cisplatin, after platination of the DNA and double-strand breaks, causes a reduction in the rate of DNA synthesis with cell-cycle arrest at G2 phase and cell death (Sorenson and Eastman 1988; Sorenson et al. 1990). However, its toxicity has been also linked to the presence of p53, as has been demonstrated by in vitro experiments (Lowe et al. 1993b).

In our in vivo investigation, the tumors induced showed a variable response: wt-p53 tumors showed a regression in size of around 60% in the first week after treatment. The mt-p53 tumors stopped growing, though no regression was observed. Thus, the response to cisplatin in vivo of tumors derived from different NSCLC lines was dependent on p53 status. Similar results have been reported in experiments utilizing oncogenically transformed fibroblasts in vivo after administration of Adriamycin (Lowe et al. 1994). This is of considerable importance because analysis of p53 status, by immunohistochemical or other methods such as the polymerase chain reaction, could make it possible to predict the response to therapy in certain patients.

We also tested the effects of radiation. The response correlated with p53 status, but its effects in wt-p53 tumors was very short, with rapid regrowth. mt-p53 tumors did not show any response, in accord with previous reports (McIlwrath et al. 1994; Kuerbitz et al. 1992). The results obtained are consistent with the clinical experience in which treatment failure is the usual pattern (Johnson et al. 1990). The actual evidence is that the p53 status does not correlate with the response to radiation.

Apoptosis, a cellular program which, when triggered, sets off a biochemical and morphologically recognizable cascade resulting in cellular suicide, was found to be responsible for the regression in wt-p53 tumors treated with cisplatin. By TUNEL assay, we detected cells with distinctive marks of apoptosis, like shrinkage, nuclear fragmentation, convulsion of the cell surface, and sequestration of the cellular content into membrane-bound apoptotic bodies (Kerr et al. 1994).

Apoptotic cells were seen in both kinds of tumor, although their frequency was directly related to p53 status. An increase of wt-p53 in apoptotic cells was evident by 24 h. Thus, apoptosis was responsible for the regression in these tumors. Interestingly, mt-p53 tumors also displayed apoptotic responses but only at high doses of cisplatin and without the same intensity. This p53-independent apoptosis has been also observed in vitro in p53-deficient mouse embryonic fibroblasts treated with high doses of chemotherapeutic drugs (Lowe et al. 1993b) and in human prostate cancer (Borner et al. 1995). Thus, it is thought cells that sustaining a sufficient amount of damage undergo apoptosis regardless of p53 status. However, in the tumors tested, this alternative route to apoptosis was not effective in producing tumoral regression. Intact p53 was necessary to achieve a favorable response.

The apoptotic response was also assessed in irradiated tumors. The increase in apoptotic cell numbers was modest and occurred very early in wt-p53 tumors. Moreover, the response varied; therefore, is doubtful that this process plays an important role in toxicity to radiation, at least in the tested cells.

The cell-cycle analysis in vivo, as in vitro, of the wt-p53 tumors and cell line showed a decrease in the percentage of S-phase cells following cisplatin treatment, suggesting arrest of the cells at the G1 check-point and apoptosis. However, in vitro we only observed a modest increase in the number of G1 cells, probably because of apoptosis. This notion is reinforced by the finding that, in mt-p53 tumors, the number of G1 cells did not change although S-phase cells accumulated. Recently, p53 has been suggested to facilitate progression through S-phase (Hawkins et al. 1996); this indicates delay at this stage and subsequent arrest in G2. Further investigation is necessary to clarify this point.

In conclusion, the results presented here show that loss of function of the p53 gene product is an important determinant of increased tumorigenicity in NSCLC tumors. In addition, the response of these tumors to cisplatin depends on the presence of this phosphoprotein, and the mechanism of the response was shown to be apoptosis. The p53-independent apoptosis, induced at high doses of cisplatin in the present study, did not control the growth of tumors. From the clinical point of view, these findings are important and complement the results of a recent clinical study in patients with NSCLC (Rusch et al. 1995). This suggested that resistance to cisplatin depends mainly on p53 status. This and previous reports support the suggestion that correction of a single genetic lesion can enhance the effect of chemotherapy.

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EXHIBIT 3

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Tapas Mukhopadhyay, *et al.*

Group Art Unit: 1642

Serial No.: 10/043,877

Examiner: B. J. Fetterolf

Filed: January 9, 2002

Att'y. Dkt. No.: INRP:095US

For: ANTIHELMINTHIC DRUGS AS A
TREATMENT FOR
HYPERPROLIFERATIVE DISEASES

SECOND DECLARATION OF TAPAS MUKHOPADHYAY, SUNIL CHADA, ABNER
MHASHILKAR, AND JACK A. ROTH UNDER 37 C.F.R. §1.131

We, Tapas Mukhopadhyay, Sunil Chada, Abner Mhashilkar, and Jack A. Roth, hereby declare as follows:

1. We are the joint inventors of the subject matter claimed in the above-referenced patent application, U.S.S.N. 10/043,887, filed January 9, 2002.

2. We are submitting this declaration to set forth facts demonstrating that we both conceived the idea of the invention as reflected in the claims of the above-referenced patent application and determined that it functioned, prior to March 9, 1999.

3. Submitted as Exhibit 1 to this declaration is a copy of a FACS assay showing our experiments and results, entitled figures "1A" and "1B" which was prepared prior to March 9, 1999.

Tapas Mukhopadhyay

8. Therefore, the invention as reflected in the claims of the above-referenced patent application was reduced to practice prior to March 9, 1999.

9. We hereby declare that all statements made by our own knowledge are true and all statements made on information and belief are believed to be true and further that statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment under § 100 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

Date

Tapas Mukhopadhyay
Tapas Mukhopadhyay

Date

Sunil Chada

Date

Abner Mhashilkar

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Jack A. Roth

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5. Exhibit 1 shows the results of our cell cycle analysis involving A549 (p53 wild type) non-small cell lung cancer (NSCLC) cells that have been treated with fenbendazole. The results show that the untreated A549 cells (A549C), have a standard profile of cells in various phases of the cell cycle, G1/S/G2, indicating a dominant G1 population. In contrast, the fenbendazole treated cells (A549 7EN) show a depression of both G2 and S phases and a G1 block. Furthermore, the fenbebdazole treated cells show a distinct sub-G0-G1 population indicative of apoptotic cells. We generated the results of this cell cycle analysis prior to March 9, 1999.

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Date

Date 07/21/05

Date

Date

Tapas Mukhopadhyay

Sunil Chada

Abner Mhashilkar

Jack A. Roth

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Sunil Chada

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08/10/05

Abhishek Mhashilkar

Date

Jack A. Roth

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Tapas Mukhopadhyay

Date

Sunil Chada

Date

Abner Mhashilkar

8-15-05

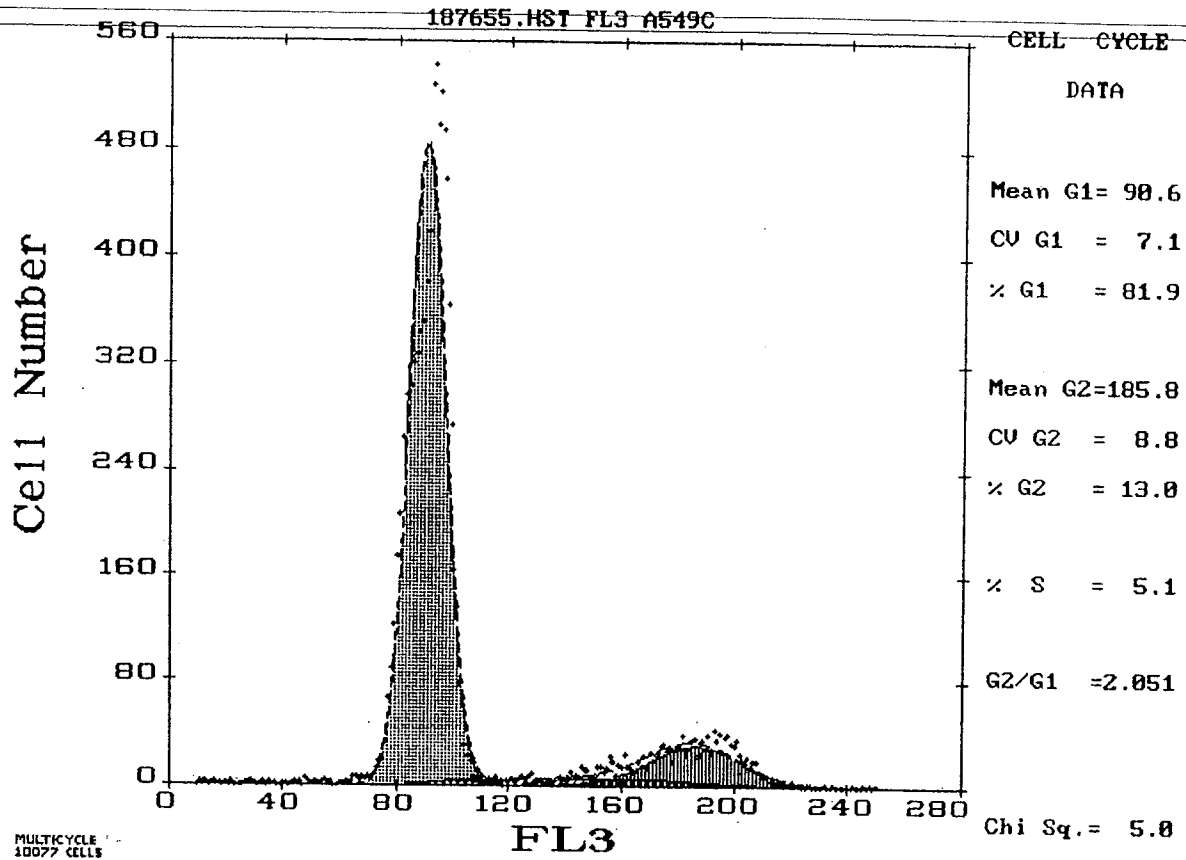
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Jack A. Roth

Exhibit 1

① A



① B

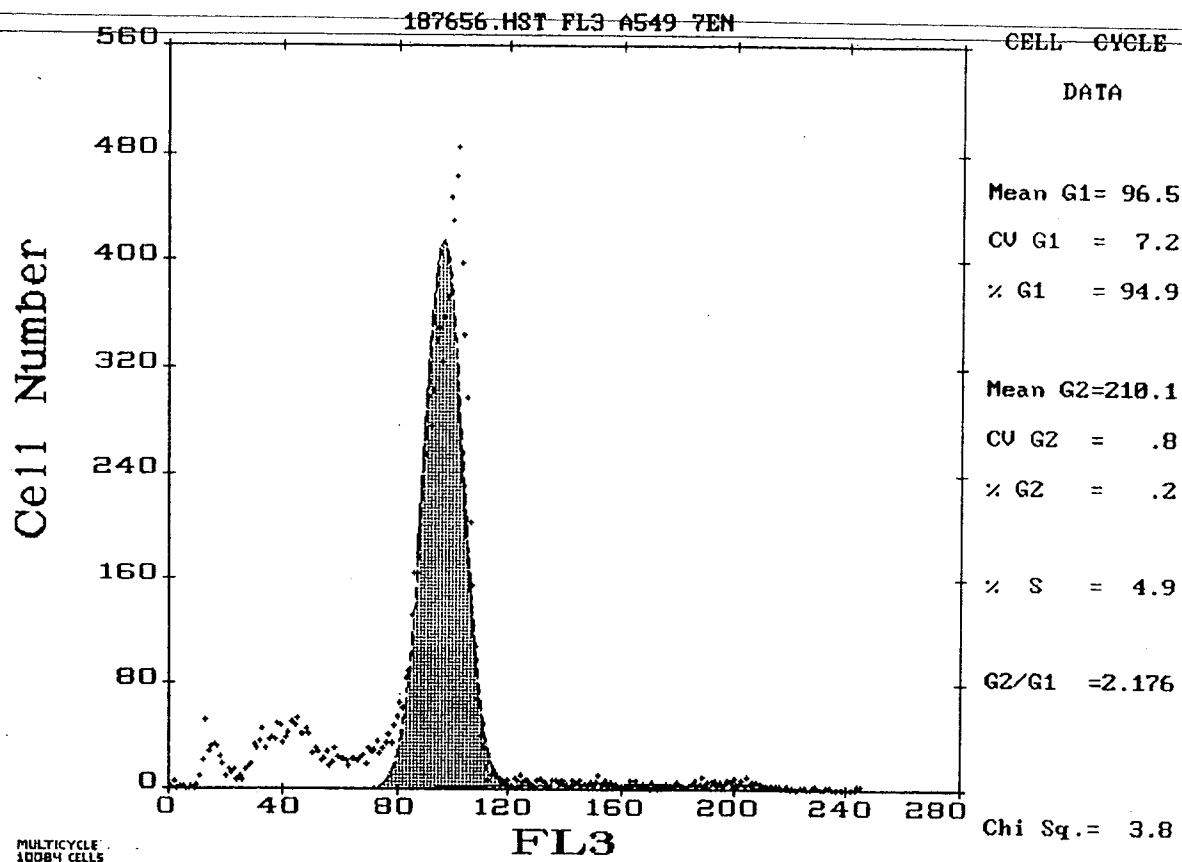
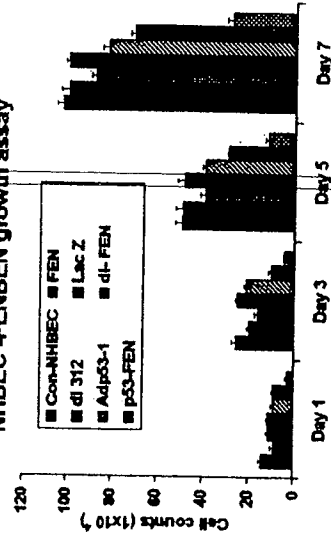
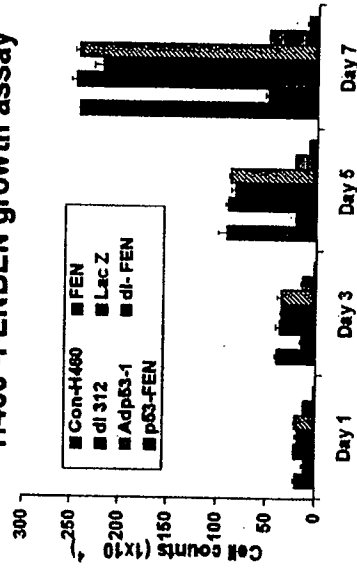
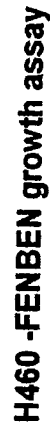
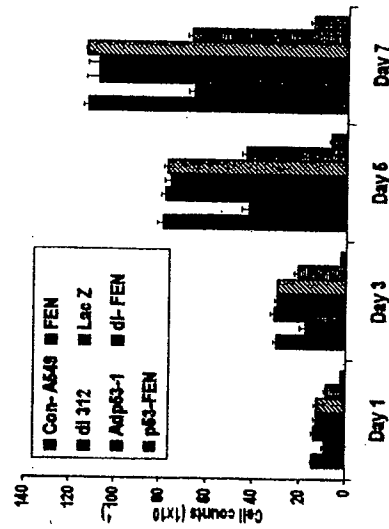
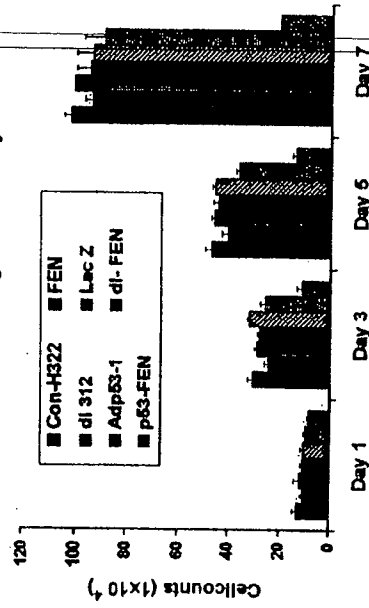
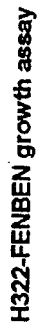
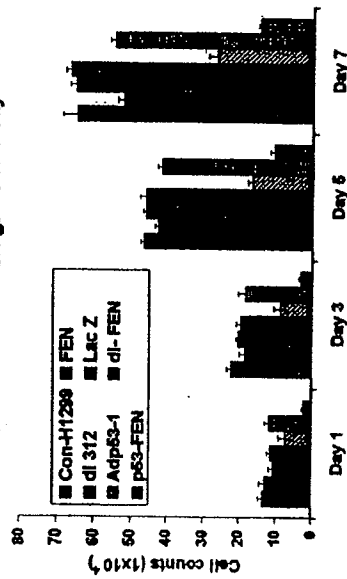
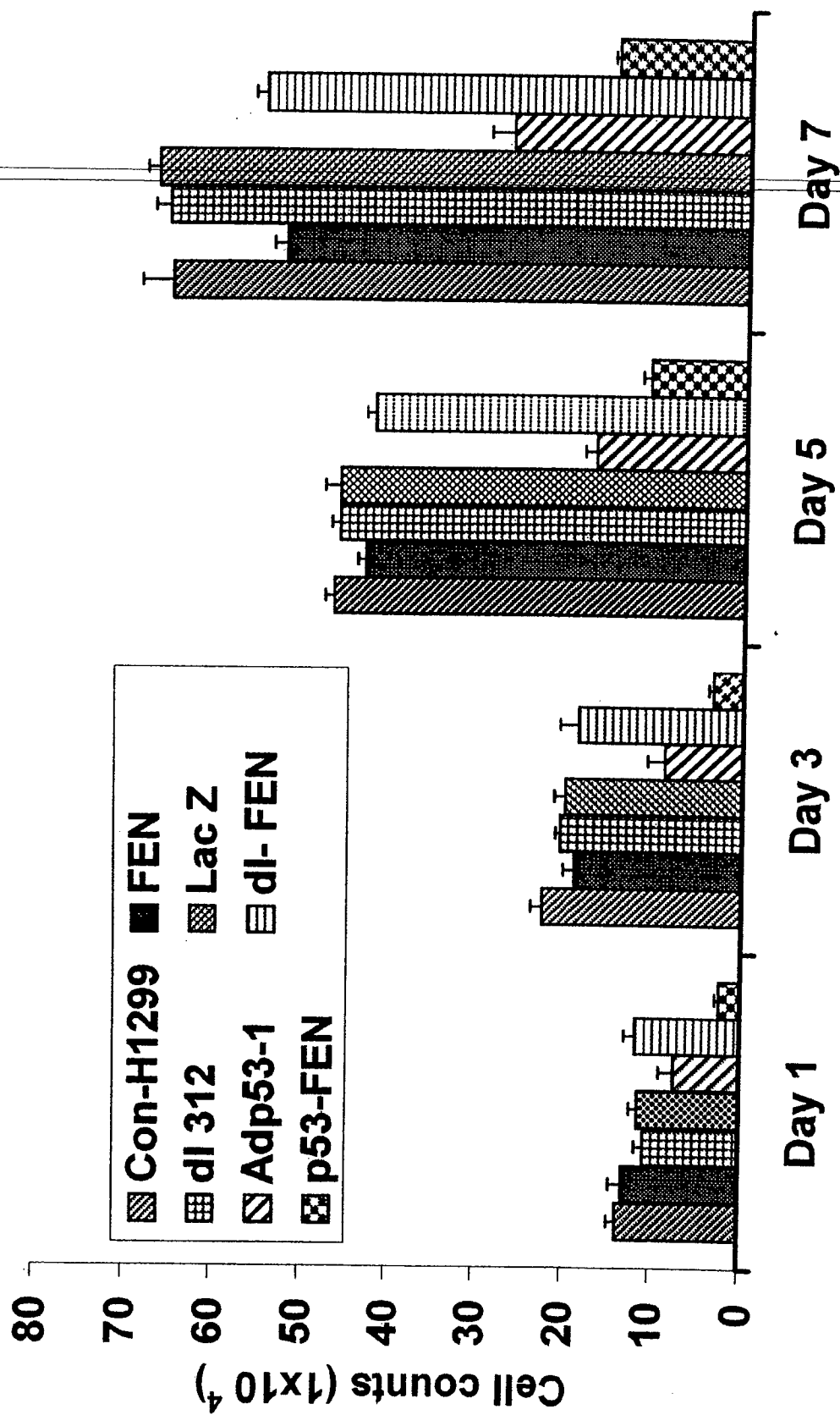


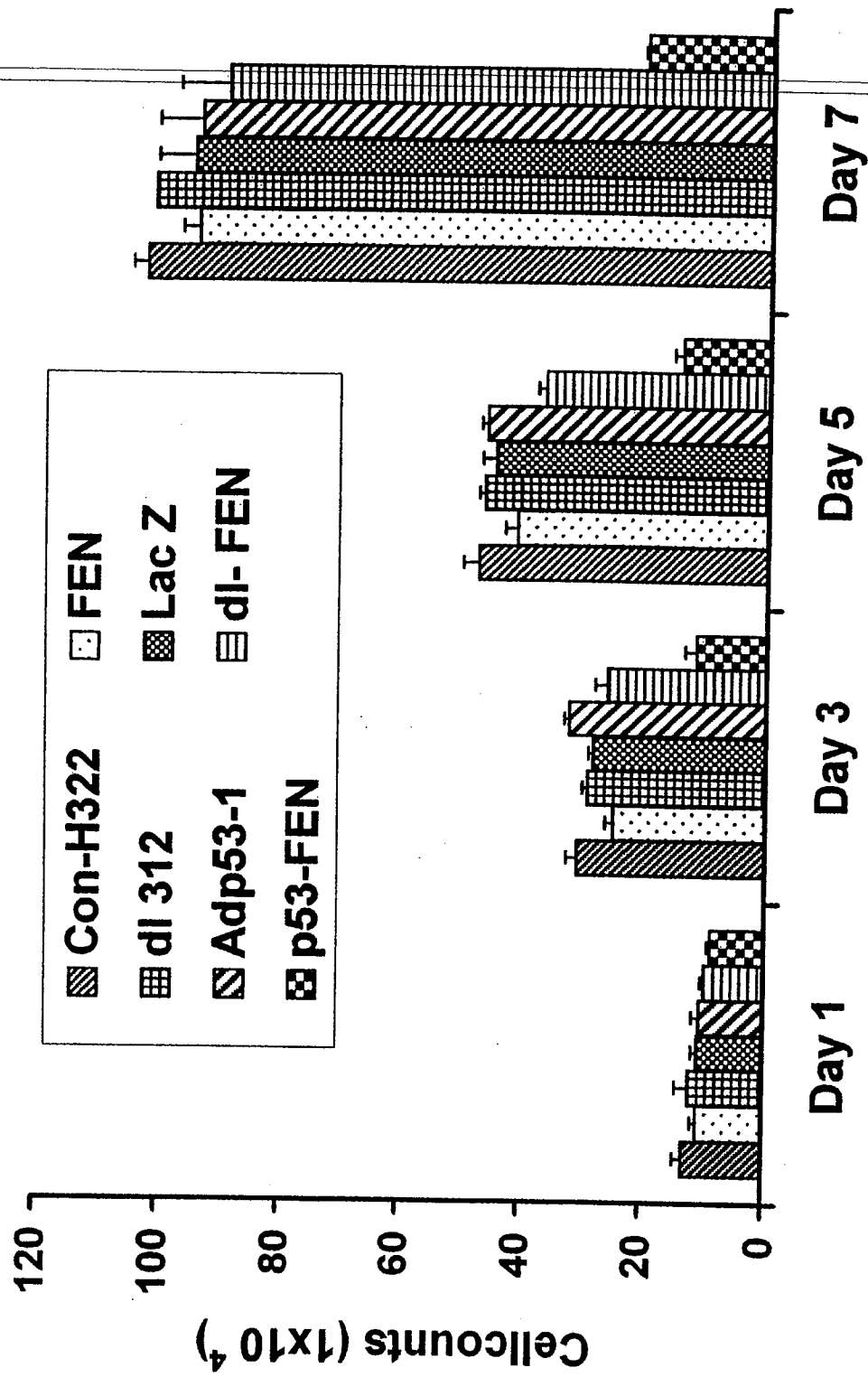
Exhibit 2



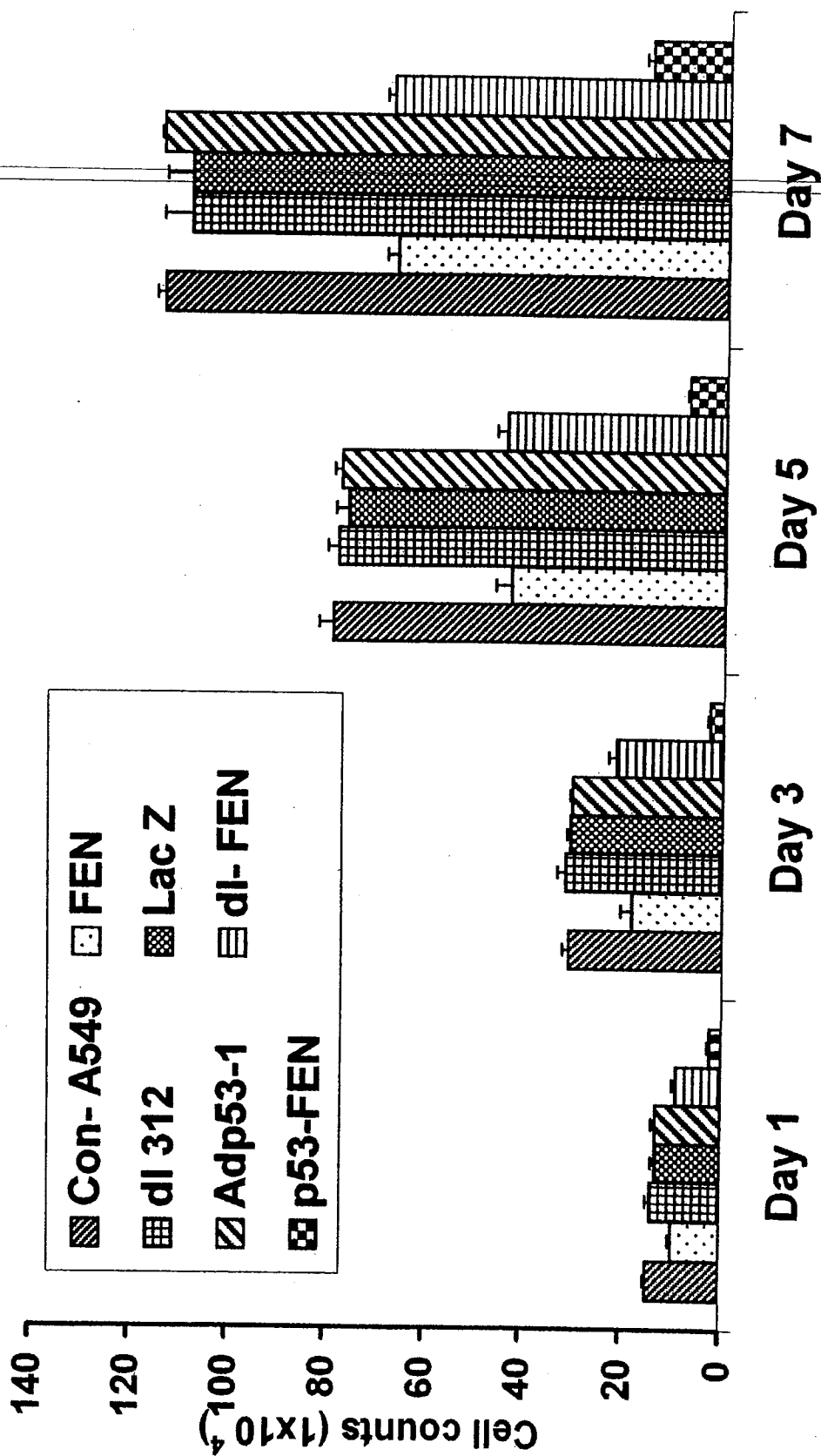
H1299-FENBEN growth assay



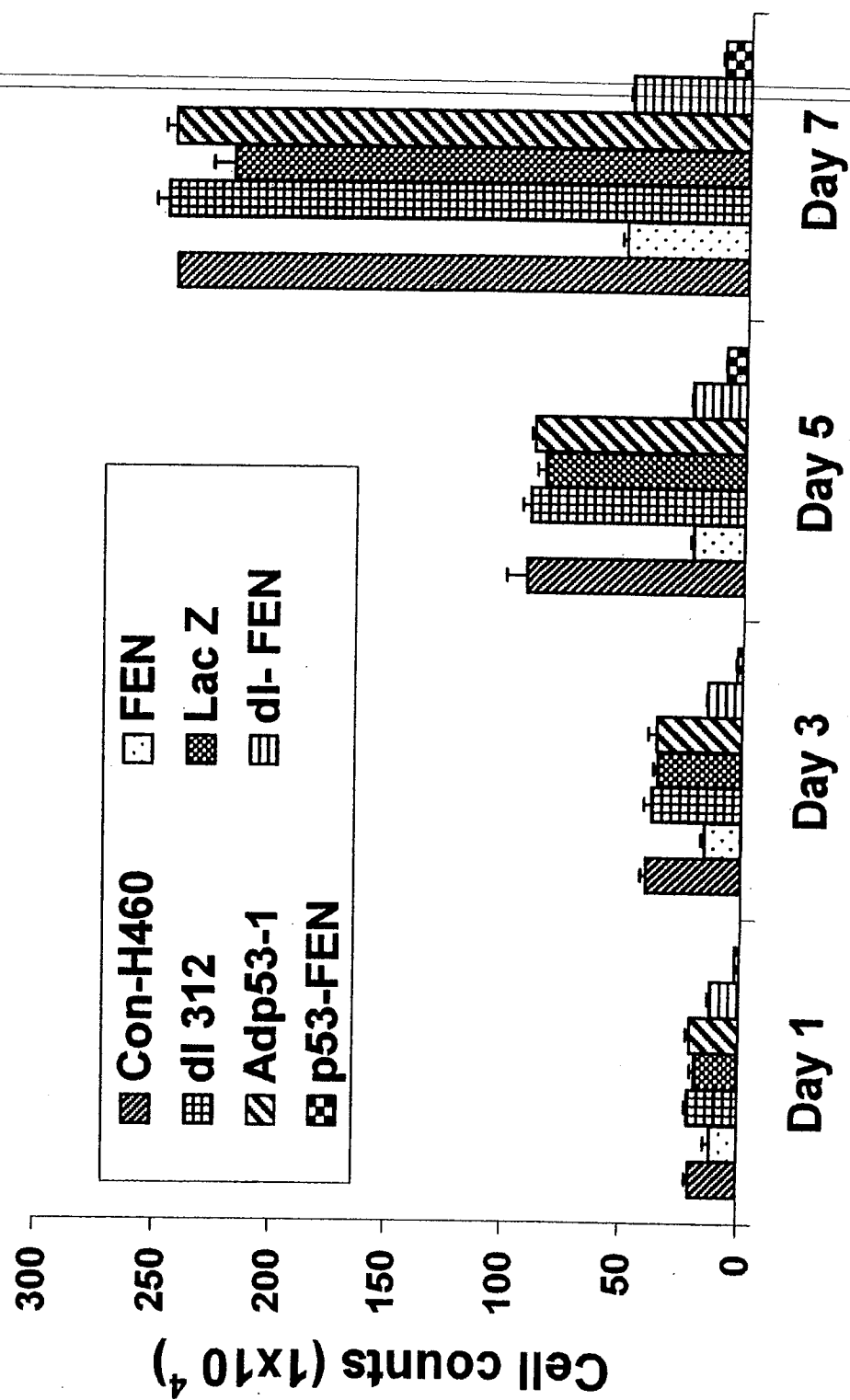
Treatment	Day 1	Day 3	Day 5	Day 7
Con-H322	~10	~25	~45	~105
dl 312	~15	~30	~40	~110
Adp53-1	~12	~28	~38	~108
p53-FEN	~10	~25	~35	~105
FEN	~8	~22	~32	~102
Lac Z	~5	~18	~28	~100
dl-FEN	~3	~15	~25	~98
p53-FEN	~2	~12	~22	~95



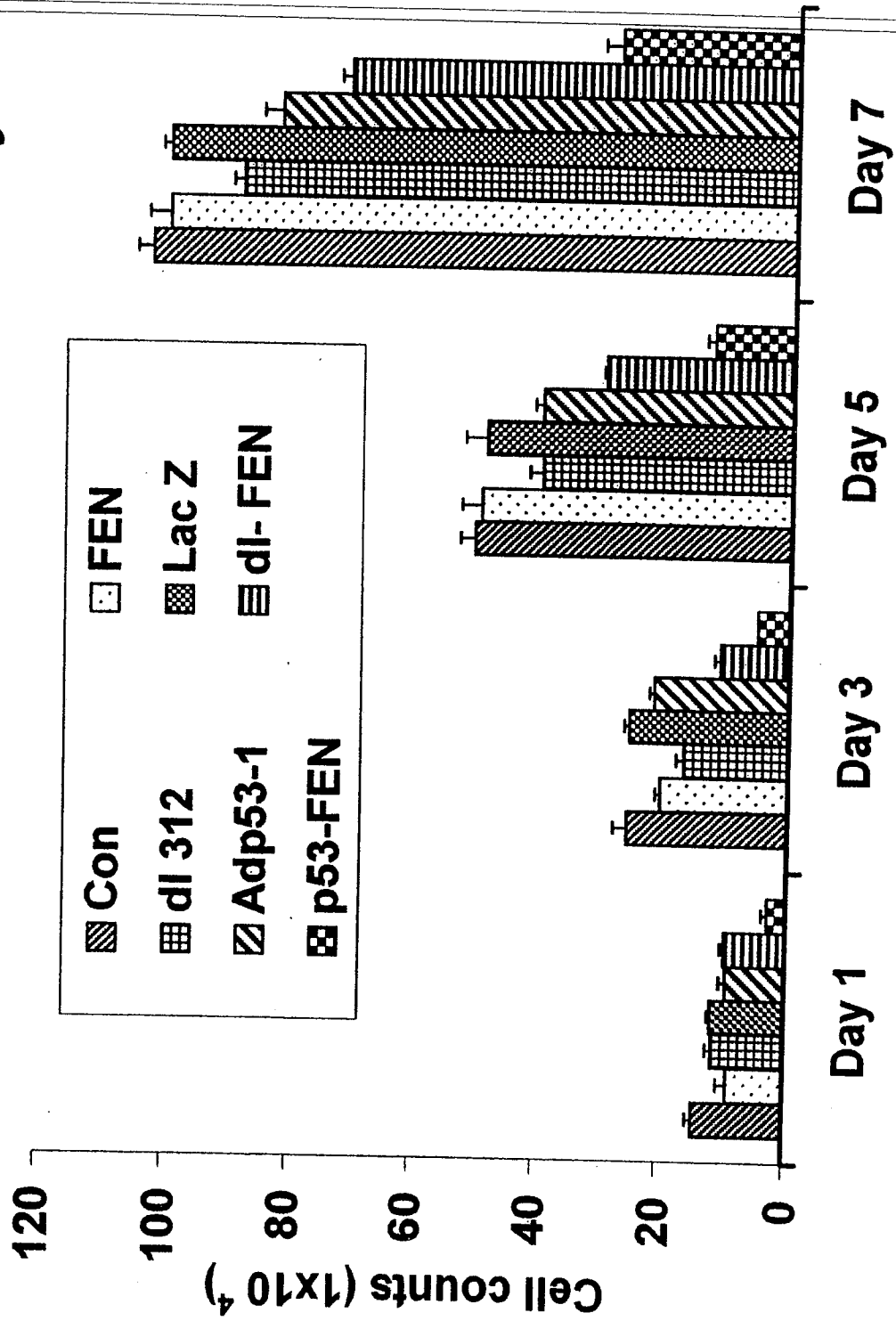
A549-FENBEN growth assay



H460 -FENBEN growth assay



HNBEC -FENBEN growth assay



NHBE

7en 105mg
 dld312 mol=1
 V3 mol=1

	Day 1		Day 3		Day 5		Day 7		9					
Control	x2	30	15	x2	50	25	x3	70	53	x5	79	44		
2		26	13		46	23		67	50		82	103		
3		30	15		56	28		62	47		84	105		
4		x2			x2			x3			x5			
5	Yes	20	10		42	21		70	53		81	101		
6		14	7		40	20		65	45		76	95		
7		19	10		38	19		60	45		82	103		
8														
9		x2			x2			x3			x5			
10	dc312	19	10		32	16		56	42		72	90		
11		24	12		30	15		52	39		69	86		
12		23	12		35	18		50	37		70	88		
13														
14		x2			x2			x3			x5			
15	Basal	22	11		48	24		71	53		80	100		
16		24	12		52	26		62	47		81	101		
17		24	12		50	25		60	45		78	98		
18														
19		x2			x2			x3			x5			
20	V3	20	10		40	20		51	38		66	83		
21		15	8		42	21		55	41		62	78		
22		19	10		44	22		52	39		68	85		
23		x2			x2			x3			x5			
24	d-F	19	10		30	10		38	29		57	71		
25		17	9		20	10		48	30		55	69		
26		20	10		24	12		39	29		58	73		
27														
28		x1			x1			x2			x3			
29		17	4		18	5		22	11		32	24		
30	V-F	10	3		19	5		27	14		39	29		
31		9	2		19	5		24	12		40	30		

EFFICIENCY LINE 22-206

H460

	Day 1		Day 3		Day 5		Day 7			
Control	25	19	x3	52	39	70	88	x10	98	245
x3	29	22		56	42	75	94		100	250
	26	20		50	38	72	90		92	230
7m	x3		x3			x3		x5		
	18	14		21	16	28	21		40	50
	17	13		19	14	30	23		38	48
	10	8		22	17	27	20		42	53
dl312	x3		x3			x5		x10		
	30	23		54	41	72	90		96	240
	27	20		44	33	68	85		98	245
	28	21		50	38	74	93		101	252
B-hal	x3		x3			x5		x10		
	21	16		49	37	62	78		82	208
	27	20		47	35	68	85		90	225
	26	20		44	33	68	85		89	223
x3	x3		x3			x5		x10		
	30	23		52	39	71	89		95	238
	27	20		41	31	70	88		97	242
	25	19		49	37	68	85		99	248
1-F	x3		x3			x3		x5		
	15	11		20	15	29	22		38	48
	18	14		20	15	29	22		40	50
	16	12		18	14	30	23		38	48
y-F	x1		x1			x2		x2		
	7	2		7	2	15	8		20	10
	8	2		10	3	17	9		20	10
	6	2		9	2	15	8		24	12

H1299

	Day 1		Day 3		Day 5		Day 7		
	x2		x2		x3		x3		
Control	25	13	47	24	60	45	80	60	
2	30	15	42	21	62	47	88	66	
3	26	13	44	22	63	47	91	68	
4	x2		x2		x3		x3		
5	30	15	34	17	58	44	67	50	
6	23	12	38	19	56	42	70	53	
7	24	12	40	20	56	42	70	53	
8									
9	x2		x2		x3		x3		
10	20	10	40	20	62	47	89	67	
11	24	12	41	21	60	45	84	63	
12	20	10	40	20	60	45	86	65	
13									
14	x2		x2		x3		x3		
15	23	12	41	21	59	44	86	65	
16	20	10	39	20	60	45	90	68	
17	24	12	36	18	64	48	88	66	
18									
19	x2		x2		x3		x3		
20	10	5	11	6	20	15	30	23	
21	15	8	19	10	22	17	36	27	
22	17	9	19	10	24	18	39	29	
23									
24	x2		x2		x3		x3		
25	20	10	32	16	55	41	72	54	
26	23	12	42	21	57	43	74	56	
27	25	13	36	18	55	41	70	53	
28	x2		x1		x2		x2		
29	9	2	10	3	20	10	30	15	
30	8	2	12	3	20	10	27	14	
31	13	3	16	4	24	12	29	15	

EFFICIENCY LINE 22-206



AS49

Central	Day 1		Day 3		Day 5		Day 7		9	
	x2		x3		x5		x5			
2	27	14	38	29	66	82	89	111		
3	30	15	42	32	60	75	90	113		
4	30	15	40	30	63	79	92	115		
5	x2		x3		x5		x5			
6	17	9	25	19	35	44	51	64		
7	19	10	27	20	36	45	52	65		
8	19	10	20	15	30	38	55	69		
9										
10	x2		x3		x5		x5			
11	27	14	38	29	60	75	80	100		
12	26	13	44	33	62	78	88	110		
13	30	15	41	31	64	80	90	113		
14										
15	x2		x3		x5		x5			
16	24	12	41	31	58	73	90	113		
17	28	14	38	29	63	79	87	109		
18	25	13	40	30	60	75	81	101		
19										
20	x2		x3		x5		x5			
21	28	14	40	30	62	78	90	113		
22	26	13	40	30	62	78	91	114		
23	24	12	39	29	60	75	90	113		
24										
25	x2		x3		x5		x5			
26	18	8	28	21	34	43	54	68		
27	17	9	30	23	37	46	52	65		
28	20	10	25	19	33	41	54	68		
29	x1		x1		x2		x3			
30	10	3	10	3	14	7	19	14		
31	8	2	12	3	16	8	20	15		
	8	2	9	2	13	7	22	17		

H3225

	Day 1		Day 3		Day 5		Day 7		
Control	x2		x3		x3		x5		
2	29	15	38	29	66	50	80	100	
3	23	12	40	30	59	44	81	101	
4	24	12	44	33	62	47	84	105	
5	x2		x3		x3		x5		
6	24	12	33	25	50	38	77	96	
7	20	10	34	26	57	43	76	95	
8	20	10	30	23	54	41	72	90	
9									
10	x2		x3		x3		x5		
11	21	11	40	30	61	46	80	100	
12	24	12	41	31	63	47	80	100	
13	26	13	35	26	60	45	82	102	
14									
15	x2		x2		x3		x5		
16	20	10	36	27	62	47	79	99	
17	20	10	37	28	58	44	78	98	
18	24	12	39	29	56	42	69	86	
19									
20	x2		x3		x3		x5		
21	20	10	41	31	62	47	82	103	
22	18	9	42	32	60	45	71	89	
23	23	12	40	30	60	45	70	88	
24									
25	x2		x3		x3		x5		
26	18	9	30	23	48	36	64	80	
27	20	10	34	26	50	38	79	99	
28	20	10	37	28	46	35	70	88	
29	x2		x2		x2		x2		
30	16	8	20	10	29	15	40	20	
31	18	9	20	10	30	15	39	20	
	18	9	27	14	24	12	41	21	

EFFICIENCY LINE 22-206



			Day 1	sd			
Con-NHB	15	13	15	14.33333	0.942809	25	23
FEN	10	7	10	9	1.414214	21	20
dl 312	10	12	12	11.33333	0.942809	16	15
Lac Z	11	12	12	11.66667	0.471405	24	26
Adp53-1	10	8	10	9.333333	0.942809	20	21
dl- FEN	10	9	10	9.666667	0.471405	10	10
p53-FEN	4	3	2	3	0.816497	5	5

			Day 1	sd			
Con-H460	19	22	20	20.33333	1.247219	39	42
FEN	14	13	8	11.66667	2.624669	16	14
dl 312	23	20	21	21.33333	1.247219	41	33
Lac Z	16	20	20	18.66667	1.885618	37	35
Adp53-1	23	20	19	20.66667	1.699673	39	31
dl- FEN	11	14	12	12.33333	1.247219	15	15
p53-FEN	2	2	2	2	0	2	3

			Day 1	sd			
Con- A549	14	15	15	14.66667	0.471405	29	32
FEN	9	10	10	9.666667	0.471405	19	20
dl 312	14	13	15	14	0.816497	29	33
Lac Z	12	14	13	13	0.816497	31	29
Adp53-1	14	13	12	13	0.816497	30	30
dl- FEN	8	9	10	9	0.816497	21	23
p53-FEN	3	2	2	2.333333	0.471405	3	3

			Day 1	sd			
Con-H322	15	12	12	13	1.414214	29	30
FEN	12	10	10	10.66667	0.942809	25	26
dl 312	11	12	13	12	0.816497	30	31
Lac Z	10	10	12	10.66667	0.942809	27	28
Adp53-1	10	9	12	10.33333	1.247219	31	32
dl- FEN	9	10	10	9.666667	0.471405	23	26
p53-FEN	8	9	9	8.666667	0.471405	10	10

			Day 1	sd			
Con-H129	13	15	13	13.66667	0.942809	24	21
FEN	15	12	12	13	1.414214	17	19
dl 312	10	12	10	10.66667	0.942809	20	21
Lac Z	12	10	12	11.33333	0.942809	21	20
Adp53-1	5	8	9	7.333333	1.699673	6	10
dl- FEN	10	12	13	11.66667	1.247219	16	21
p53-FEN	2	2	3	2.333333	0.471405	3	3

	Day 3	sd
28	25.33333	2.054805
19	20	0.816497
18	16.33333	1.247219
25	25	0.816497
22	21	0.816497
12	10.66667	0.942809
5	5	0

53	50
53	49
42	39
53	47
38	41
29	30
11	14

	Day 5	sd
47	50	2.44949
45	49	3.265986
37	39.33333	2.054805
45	48.33333	3.399346
39	39.33333	1.247219
29	29.33333	0.471405
12	12.33333	1.247219

	Day 3	sd
38	39.66667	1.699673
17	15.66667	1.247219
38	37.33333	3.299832
33	35	1.632993
37	35.66667	3.399346
14	14.66667	0.471405
2	2.333333	0.471405

88	94
21	23
90	85
78	85
89	88
22	22
8	9

	Day 5	sd
90	90.66667	2.494438
20	21.33333	1.247219
93	89.33333	3.299832
85	82.66667	3.299832
85	87.33333	1.699673
23	22.33333	0.471405
8	8.333333	0.471405

	Day 3	sd
30	30.33333	1.247219
15	18	2.160247
31	31	1.632993
30	30	0.816497
29	29.66667	0.471405
19	21	1.632993
2	2.666667	0.471405

82	75
44	45
75	78
73	79
78	78
43	46
7	8

	Day 5	sd
79	78.66667	2.867442
38	42.33333	3.091206
80	77.66667	2.054805
75	75.66667	2.494438
75	77	1.414214
41	43.33333	2.054805
7	7.333333	0.471405

	Day 3	sd
33	30.66667	1.699673
23	24.66667	1.247219
26	29	2.160247
29	28	0.816497
33	32	0.816497
28	25.66667	2.054805
14	11.33333	1.885618

50	44
38	43
46	47
47	44
47	45
36	38
15	15

	Day 5	sd
47	47	2.44949
41	40.66667	2.054805
45	46	0.816497
42	44.33333	2.054805
45	45.66667	0.942809
35	36.33333	1.247219
12	14	1.414214

	Day 3	sd
22	22.33333	1.247219
20	18.66667	1.247219
20	20.33333	0.471405
18	19.66667	1.247219
10	8.666667	1.885618
18	18.33333	2.054805
4	3.333333	0.471405

45	47
44	42
47	45
44	45
15	17
41	43
10	10

	Day 5	sd
47	46.33333	0.942809
42	42.66667	0.942809
45	45.66667	0.942809
48	45.66667	1.699673
18	16.66667	1.247219
41	41.66667	0.942809
12	10.66667	0.942809

		Day 7	sd
99	103	105 102.3333	2.494438
101	95	103 99.66667	3.399346
90	86	88 88	1.632993
100	101	98 99.66667	1.247219
83	78	85 82	2.94392
71	69	73 71	1.632993
24	29	30 27.66667	2.624669

		Day 7	sd
245	250	230 241.6667	8.498366
50	48	53 50.33333	2.054805
240	245	252 245.6667	4.921608
205	225	223 217.6667	8.993825
238	242	248 242.6667	4.109609
48	50	48 48.66667	0.942809
10	10	12 10.66667	0.942809

		Day 7	sd
111	113	115 113	1.632993
64	65	69 66	2.160247
100	110	113 107.6667	5.557777
113	109	101 107.6667	4.988877
113	114	113 113.3333	0.471405
68	65	68 67	1.414214
14	15	17 15.33333	1.247219

		Day 7	sd
100	101	105 102	2.160247
96	95	90 93.66667	2.624669
100	100	102 100.6667	0.942809
99	98	86 94.33333	5.906682
103	89	88 93.33333	6.847546
80	99	88 89	7.788881
20	20	21 20.33333	0.471405

		Day 7	sd
60	66	68 64.66667	3.399346
50	53	53 52	1.414214
67	63	65 65	1.632993
65	68	66 66.33333	1.247219
23	27	29 26.33333	2.494438
54	56	53 54.33333	1.247219
15	14	15 14.66667	0.471405

APPENDIX B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Tapas Mukhopadhyay, *et al.*

Serial No.: 10/043,877

Filed: January 9, 2002

For: ANTIHELMINTHIC DRUGS AS A
TREATMENT FOR
HYPERPROLIFERATIVE DISEASES

Group Art Unit: 1642

Examiner: B. J. Fetterolf

Atty. Dkt. No.: INRP:095US

**THIRD DECLARATION OF TAPAS MUKHOPADHYAY, SUNIL CHADA,
ABNER MHASHILKAR, AND JACK A. ROTH UNDER 37 C.F.R. §1.131**

We, Tapas Mukhopadhyay, Sunil Chada, Abner Mhashilkar, and Jack A. Roth,
hereby declare as follows:

1. We are the joint inventors of the subject matter claimed in the above-referenced patent application, U.S.S.N. 10/043,887, filed January 9, 2002.

2. We previously submitted a declaration to set forth facts demonstrating that we both conceived the idea of the invention as reflected in the claims of the above-referenced patent application and determined that it functioned, prior to March 9, 1999.

3. In the present declaration we are submitting facts demonstrating that we steadily progressed in our research to confirm that our invention functioned in an animal model, which was in accordance with our initial understanding. We continually and

diligently conducted these studies from the time we conceived of our invention until the time our U.S. Provisional patent application, U.S.S.N. 60/261,346, was filed on January 11, 2001. Evidence of our diligence is set forth in Exhibits 1-3, discussed in detail below.

4. Submitted as Exhibit 1 to this declaration is a copy of a draft manuscript of our experiments and results, entitled "Potent Induction of Apoptosis by Anthelmintics in Human Lung Cancer Cells: Involvement of Wild-Type p53 and p21 Kinase Inhibitor." The studies set forth in this manuscript and the preparation of this manuscript took place prior to January 14, 2000.

5. Submitted as Exhibit 2 to this declaration is a copy a series of experiments and results involving the use of benzimidazoles in the treatment of p53 wild type expressing tumor cells, ending with animal models, as evidenced by the laboratory notebook of Dr. Jiichiro Sasaki, who worked under the direction of Dr. Tapas Mukhopadhyay. These experiments took place between November 10, 2000 and September 9, 2001.

6. Submitted as Exhibit 3 to this declaration is a copy of a draft manuscript of our experiments and results, including in animal models, entitled "Mebendazole: A Novel Microtubule Agent Having Potent Antitumor Activity," which was submitted for publication on October 25, 2001.

7. Exhibit 2 shows the preparation and results of a series of experiments pertaining to the benzimidazole drug, mebendazole (labeled MZ) for the treatment of cancer. Experiments pertaining to the treatment of cancer cells with mebendazole are listed on the following dates: November 11, 2000; November 15, 2000; December 5, 2000; December 8, 2000; December 12, 2000; January 11, 2001; January 12, 2001; January 18, 2001; February 10, 2001; February 20, 2001; February 23, 2001; February 26, 2001; March 2-6, 2001; March 19, 2001; March 20, 2001; March 29-31, 2001; April 3-4, 2001, April 14, 2001; April 19, 2001; April 26, 2001; June 6, 2001, June 7, 2001; June 16, 2001; July 24, 2001; July 26-28, 2001; August 1, 2001; August 4, 2001; August 5, 2001; August 7, 2001; August 31, 2001; August 31, 2001 and September 9, 2001. Of note, the last two entries pertain to the use of mebendazole in animal models.

8. Exhibit 3 shows the draft manuscript pertaining to the treatment of cancer cells with mebendazole on the inhibition of human tumor xenografts in mice. See Exhibit 3, Abstract, page 2. In accordance with our in vitro data, our animal model data showed that Mebendazole inhibited lung cancer growth. For example oral administration of mebendazole to mice previously injected with A549 lung cancer cells resulted in an 80% reduction in tumor count as compared to the control. See Exhibit 3, Results, page 10-11.

9. All work disclosed in the invention disclosure form was conducted in the United States of America.

10. Therefore, the invention as reflected in claims 75-77, 83-106, 161-162 and 184 of the above-referenced patent application was conceived of prior to March 9, 1999 and diligently reduced to practice.

11. We hereby declare that all statements made by our own knowledge are true and all statements made on information and belief are believed to be true and further that statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment under § 100 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

_____ Date	_____ Tapas Mukhopadhyay
_____ Date	_____ Sunil Chada
_____ Date	_____ Abner Mhashilkar
_____ Date	_____ Jack A. Roth

EXHIBIT 4



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/043,877	01/09/2002	Tapas Mukhopadhyay	INRP:095US 10200175	6285
7590 11/16/2005 FULBRIGHT & JAWORSKI L.L.P. SUITE 2400 600 CONGRESS AVENUE AUSTIN, TX 78701			EXAMINER FETTEROLF, BRANDON J	
			ART UNIT 1642	PAPER NUMBER

DATE MAILED: 11/16/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

FULBRIGHT & JAWORSKI, LLP
IPT DOCKETING
Docketed ☒ Not Req'd ☐ Confirmation ☐
Initials 1st [Signature] Initials 2nd [Signature]
NOV 21 2005
Attorney [Signature]
Docket No. INRP:095US
Action Req'd _____ Date Due _____

3 mo OA initial deadline
2/16/06. Final 5/16/06.

Office Action Summary

Application No.

10/043,877

Applicant(s)

MUKHOPADHYAY ET AL.

Examiner

Brandon J. Fetterolf, PhD

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-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 22 August 2005.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 1-163, 166, 168, 171-175, 183 and 184 is/are pending in the application.

4a) Of the above claim(s) 4-8, 11, 30-74, 78-82, 107-160, 163, 166, 168 and 171-175 is/are withdrawn from consideration.

- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 1-3, 9, 12-19, 21-29, 75-77, 83-97, 99-106, 161, 162, 183 and 184 is/are rejected.
- 7) ☒ Claim(s) 10, 20 and 98 is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. _____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- 1) ☒ Notice of References Cited (PTO-892)
- 2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
- 3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08)
Paper No(s)/Mail Date _____
- 4) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____
- 5) ☐ Notice of Informal Patent Application (PTO-152)
- 6) ☐ Other: _____

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Mukhopadhyay et al.

Response to the Amendment

The Amendment filed on 08/22/2005 in response to the previous Non-Final Office Action (03/18/2005) is acknowledged and has been entered.

Claims 164-165, 167, 169-170, 176-182 have been canceled.

Claims 1-163, 166, 168, 171-175 and 183-184 are currently pending.

Claims 4-8, 11, 30-74, 78-82, 107-160, 163, 166, 168 and 171-175 are withdrawn from consideration as being drawn to a non-elected invention and/or species.

Claims 1-3, 9-10, 12-29, 75-77, 83-106, 161-162 and 183-184 are currently under consideration.

The Declaration Under CFR 1.131 filed on 08/22/2005 by the inventors is acknowledged and has been considered. The Declaration filed on 08/22/2005 under 37 CFR 1.131 is sufficient to overcome the Camden et al reference with respect to claims 1-3, 9-10 and 12-29 as specifically drawn to a method of inducing apoptosis in a cell expressing a tumor suppressor gene comprising administering an effective amount of a benzimidazole to said cell, wherein the expression of the tumor suppressor gene by the cell and the benzimidazole results in the apoptosis of the cell. However, the evidence submitted is insufficient to establish a reduction to practice of the invention in this country or a NAFTA or WTO member country prior to the effective date of the Camden reference with respect to claims 75-77, 83-106, 161-162 and 184. As noted above, the Declaration clearly shows reduction to practice of an *in vitro* method of inducing apoptosis but appears to be silent of the reduction to practice of the presently claimed *in vivo* methods recited in claims 75-77, 83-106, 161-162 and 184. While Applicants contend (Remarks, page 22) that reduction to practice is shown by the fact that the cell types used in the experiments were human cells, those of skill in the art recognize the unpredictability of extrapolating in vitro data to in vivo (see for example, Freshney (Culture of Animal Cells, A Manual of Basic Technique, Alan R. Liss, Inc., 1983, New York, p4); Dermer (Bio/Technology, 1994, 12:320)). Moreover, Applicants contend that the law is clear that a Rule 131 Declaration need only show as much as the prior art discloses. However, contrary to

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Applicants assertion, Camden discloses *in vivo* treatment (beginning on column 14, line 53 to column 25, line 6). As such, the Declaration is insufficient to overcome the Camden et al. reference with respect to these claims.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office Action.

Rejections Maintained:

Claims 75-76, 83-97 and 99-100 **remain** rejected under 35 U.S.C. 102(e) as being anticipated by Camden (US 6,262,093, 1999).

Camden teaches (column 11, line 69 to column 12, line 51) a method of inducing apoptosis in cancer cells expressing abnormal p53 by administering an effective amount of a benzimidazole derivative. The patent further teaches (column 12, line 52 to column 13, line 24) a method of treating a patient having cancer expressing abnormal p53 by administering an effective amount of a benzimidazole derivative to induce apoptosis. Moreover, Camden discloses (column 14, line 53 to column 24, line 31) a method of treating a patient with cancer comprising administering an effective amount of a benzimidazole derivative. With regards to the cancer, the patent teaches that cancer includes, but is not limited to, cancers of the breast, lung, non-small cell lung and sarcoma (column 3, lines 45-50) or cancer that has survived treatment with another anticancer agent (column 29, lines 9-13). Specifically, Camden discloses the apoptotic effect in cancer cells such as, for example, MCF7 breast cells both *in vitro* (column 12, lines 46-51) and *in vivo* (column 16, lines 48+). With regards to the cancer cells, the patent teaches (column 12, lines 46-51) that some of the cancer cell lines tested are known to express abnormal p53. With regards to administration, Camden provides that 1 to 1000 mg/kg of a benzimidazole derivative (column 5, line 58 to column 6, line 17) can be administered orally, by intravenous injection, by parental administration or by injection into or around the tumor (column 6, lines 26-43). In addition, Camden teaches that the compound can be administered as a single daily dose or repeated at least once (column 6, lines 18-25). Furthermore, the patent shows that even at a concentration less than 10 $\mu\text{g/mL}$, the benzimidazole derivatives were capable of inducing apoptosis in p53 abnormal cell lines (column 12, lines 46-51). Thus, while

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Camden does not characterize the breast cells as expressing the tumor suppressor gene MDA-7, the claimed functional limitation would be an inherent property of the referenced since the specification (page 14, lines 11-18) teaches that MDA-7 is expressed in human breast cancer cells. Thus, it does not appear that the claim language or limitation results in a manipulative difference in the method steps when compared to the prior art disclosure. See Bristol-Myers Squibb Company v. Ben Venue Laboratories 58 USPQ2d 1508 (CAFC 2001).

In reference to the rejection, Applicants contend (Remarks, beginning on page 16) that Camden fails to disclose each limitation of the claimed invention because it fails to expressly or inherently disclose the limitation “wherein the expression of a tumor suppressor gene by the cell and the benzimidazole results in inhibition of said cancer.” For examples, Applicants argue that the sections of Camden cited by the Examiner do not appear to include any information whatsoever regarding the expression of a tumor suppressor gene, or any information correlating expression of a tumor suppressor gene with inhibition of cancer. Specifically, Applicants assert that the section cited by the Examiner (column 11, line 69 to column 12, line 51) is directed to cells expressing abnormal p53 (see, e.g., column 12, lines 49-51). Moreover, Applicants submit that Camden does not provide any information regarding measuring p53 protein or mRNA, or any results correlating p53 expression with inhibition of cancer. Furthermore, Applicants contend that Camden presents no information comparing p53 expressing cells with cells that do not express any p53. With regards to inherency, Applicants argue that for inherent anticipation to arise “the prior art necessarily function in accordance with or includes, the claimed limitation.” *Atlas Powder Co.*, 190 F.3d at 1347. (citing *In re King*, 801 F.2d 1324, 11326 (Fed. Cir., 1986). For example, Applicants assert that in order for Camden to fulfill inherent anticipation there must be, at the very least, expression of a tumor suppressor gene upon apoptotic cell death. Applicants submit that Camden does not appear to teach any such requirement, but instead teaches that a normal p53 gene is not required for apoptosis to occur. Moreover, Applicants submit that Camden does not anticipate the invention because it does not disclose the limitation “wherein the tumor cell is a multidrug resistant tumor cell.” For example, Applicants argue that while Camden discloses the treatment of cancer with one of the specifically defined benzimidazole derivatives wherein the patient has survived treatment with another anticancer agent, Applicants contend that this is distinguishable from a tumor cell exhibiting the properties of multidrug resistance. Furthermore, Applicants submit that Camden fails to

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disclose any cancer cells expressing the tumor suppressor gene MDA-7. For example, Applicants contend that while the Examiner states that all breast cancer cells express MDA-7, Applicants assert that the Examiner misinterpreted the information provided in the specification of the present invention (page 14, lines 11-18) because the statement is made in reference to Su et al. entitled, "The cancer growth suppressor gene mda-7 selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice," which indicates that when breast cancer cells are infected with adenoviral vectors expressing the MDA-7 gene, the expression of this gene induces apoptosis.

These arguments have been carefully considered, but are not found persuasive.

The previous rejection was based on the technical reasoning that necessarily flowed from the prior art; e.g., a method of treating a patient having cancer expressing abnormal p53 by administering an effective amount of a benzimidazole derivative to inhibit cancer. Thus, while Applicants contend that Camden fails to disclose each limitation of the claimed invention because it fails to expressly or inherently disclose the limitation "wherein the expression of a tumor suppressor gene by the cell and the benzimidazole results in inhibition of cancer", Applicants have not clearly provided evidence or patentable difference between the instantly claimed method and that of the prior art. For example, as stated by Applicants Camden is "directed to cells expressing abnormal p53 (see, e.g., column 12, lines 49-51)". Thus, it appears that the Camden teaches cells that express abnormal p53. Moreover, in response to Applicants contention that Camden does not teach expression of a tumor suppressor gene by the cell and the benzimidazole results in inhibition of cancer, the Examiner recognizes that even though the claims are drawn to a mechanism by which cancer cells are inhibited, the claimed method does not appear to distinguish over the prior art teaching of the same or nearly the same method. For example, the specification discloses (page 65, Example 3) experiments performed *in vivo* to evaluate the anti-tumor activity of mebendazole in mice inoculated with A549 tumor cells, while Camden teaches a method of reducing tumor growth in mice models using tumor cell lines such as MXI (breast), A549 (lung) and HT29 (colon (column 23, lines 43-60). With regards to the HT29 cell line, the patent teaches that HT29 cells comprise abnormal p53 (column 11, line 65 to column 12, line 19). With regards to A549 cell line, the specification teaches (page 64, Table 4) that A549 cell line expresses wild type p53. As such, the mechanism of action does not have a bearing on the patentability of the invention if the invention was already known or obvious. Mere recognition of latent properties in the prior art does not render

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nonobvious an otherwise known invention. In re Wiseman, 201 USPQ 658 (CCPA 1979).

Granting a patent on the discovery of an unknown but inherent function would remove from the public that which is in the public domain by virtue of its inclusion in, or obviousness from, the prior art. In re Baxter Travenol Labs, 21 USPQ2d 1281 (Fed. Cir. 1991). See M.P.E.P. 2145. Moreover, the office does not have the facilities and resources to provide the factual evidence needed in order to establish that the product of the prior art does not possess the same material, structural and functional characteristics of the claimed product. In the absence of evidence to the contrary, the burden is on the applicant to prove that the claimed product is different from those taught by the prior art and to establish patentable differences. See In re Best 562F.2d 1252, 195 USPQ 430 (CCPA 1977) and Ex parte Gray 10 USPQ 2d 1922 (PTO Bd. Pat. App. & Int. 1989). In addition, in contrast to Applicants contention that Camden does not teach any information regarding measuring p53 protein or mRNA, any results correlating p53 expression with inhibition of cancer or comparing p53 expressing cells with cells that do not express any p53, the instant claims do not appear to differentiate between abnormal or wild-type tumor suppressor expression, nor do the claims which have been rejected require any type of measurement. Furthermore, Applicants argument that Camden does not anticipate the invention because multidrug resistant tumor cells exhibit properties which differ from those that have been exposed to another anticancer agent is not pertinent because Applicants have not provided any factual evidence to support these allegations. What is to say that a tumor cell which have been treated previously with chemotherapeutics have not developed resistance? The office does not have the facilities and resources to provide the factual evidence needed in order to establish that the product of the prior art does not possess the same material, structural and functional characteristics of the claimed product. In the absence of evidence to the contrary, the burden is on the applicant to prove that the claimed product is different from those taught by the prior art and to establish patentable differences. See In re Best 562F.2d 1252, 195 USPQ 430 (CCPA 1977) and Ex parte Gray 10 USPQ 2d 1922 (PTO Bd. Pat. App. & Int. 1989). Lastly, in response to Applicants assertion that the Examiner misinterpreted the information provided in the specification of the present invention regarding MDA-7 (page 14, lines 11-18), the Examiner recognizes this misinterpretation and agrees with Applicants interpretation of *Su et al.* However, while *Su et al.* indicates breast cancer cells which are infected with adenovirus vectors expressing MDA-7 gene, the reference clearly sets forth that MDA-7 is expressed in metastatic

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melanoma (see page 14400, 2nd column, 2nd full paragraph). Thus, in view of this and the Camden method of administering a benzimidazole derivative for the treatment of melanoma (column 23, lines 43-60), it appears that the expression of MDA-7 is an inherent property of melanoma cells. Therefore, claims 75-76, 83-100 remain rejected under 35 U.S.C. 102(e) as being anticipated by Camden (US 6,262,093, 1999)

Claims 75-76, 83-97 and 99-106 **remain** rejected under 35 U.S.C. 103(a) as being unpatentable over Camden (US 6,262,093, 1999) in combination with Perdoma *et al.* (J. Cancer Res. Clin. Oncol. 1998, 124, 10-18).

Camden teaches as set forth above with regard to claims 75-76, 83-97 and 99-100, a method of treating cancer by inducing apoptosis to a cell expressing abnormal p53 comprising administering a benzimidazole derivative.

Camden does not teach determining the tumor suppressor status by way of Southern blotting, Northern blotting, PCR, ELISA or Western blotting (claims 23-28 and 101-106).

Perdoma *et al.* teach determining the p53 status, by Western blot analysis (page 12, 3rd paragraph) or other methods such as polymerase chain reaction (PCR), could make it possible to predict the response to therapy in certain patients (page 17, 1st column, 2nd paragraph). Perdoma *et al.* further teach that the response to cisplatin *in vivo* of NSCLC tumor lines was dependent on p53 status (page 17, 1st column, 2nd paragraph). Specifically, the reference teaches wt-p53 tumors showed a regression in size of around 60%, whereas mt-p53 tumors stopped growing (page 17, 1st column, 2nd paragraph).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to determine the status of a tumor suppressor gene, like p53, in a tumor cell prior to administering a benzimidazole derivative using techniques such as Western blot, PCR or other methods of analysis. One would have been motivated to do so because Camden teaches the selectivity in killing p53 abnormal cell lines versus cells expressing normal p53 (column 12, lines 52+), while Perdoma *et al.* teaches that the “response to cisplatin *in vivo* of tumors derived from different NSCLC lines was dependent on p53 status (page 17, 1st column, 2nd paragraph).” Further, one of ordinary skill in the art would have a reasonable expectation of success because Perdoma *et al.* teaches “analysis of p53 status, by immunohistochemical or other methods such as the

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polymerase chain reaction (PCR), could make it possible to predict the response to therapy in certain patients (page 17, 1st column, 2nd paragraph)."

In reference to the rejection, Applicants contend that a prima facie case of obviousness has not been established because the prior art reference cited by the Examiner do not teach or suggest all of the claim limitation. For the reasons set forth above, Applicants contend that the Examiner has not shown that Camden teaches or suggest inhibition of cancer as the result of expression of a tumor suppressor gene and the administration of a benzimidazole. Nor does Camden teach the particular benzimidazoles found in claims 161-162 and 183 or the limitation "wherein the tumor cell is a multidrug resistant tumor cell. Moreover, Applicants argue that Perdomo does not teach or suggest the missing limitations that are not disclosed in Camden. For example, Applicants assert that the Examiner has not shown where Perdomo includes any information pertaining to benzimidazoles, or the effect of benzimidazoles on tumor cells, nor do Applicants find any such disclosure in Perdomo. Lastly, Applicants argue that Perdomo provides no motivation to one of ordinary skill in the art to provide the limitation since it does not even address benzimidazoles.

These arguments have been carefully considered, but are not found persuasive.

First, Applicants arguments pertaining to Camden have been addressed by the Examiner above and have been incorporated herein. Secondly, while the Examiner concedes that Perdomo does not teach any information pertaining to benzimidazoles, or the effect of benzimidazoles on tumor cells, Applicants appear to be considering the references individually. However, the courts have held that one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references. In *re Keller*, 642 F.2d 13, 208 USPQ 871 (CCPA 1981); In *re Merck & Co., Inc.*, 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986). In response to Applicants argument that there is no suggestion to combine the references, the examiner recognizes that obviousness can only be established by combining or modifying the teachings of the prior art to produce the claimed invention where there is some teaching, suggestion, or motivation to do so found either in the references themselves or in the knowledge generally available to one of ordinary skill in the art. See *In re Fine*, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988) and *In re Jones*, 958 F.2d 347, 21 USPQ2d 1941 (Fed. Cir. 1992). In this case, the motivation lies within Perdomo whom teaches that the analysis of p53 status could make it possible to predict the response to therapy in certain patients. As such, claims 75-76, 83-106, 161-162 remain and new claim 183 is rejected under

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35 U.S.C. 103(a) as being unpatentable over Camden (US 6,262,093, 1999) in combination with Perdoma *et al.* (J. Cancer Res. Clin. Oncol. 1998, 124, 10-18).

Claims 75-77, 83-97, 99 and 161-162 **remain** and **new** claim 184 is rejected under 35 U.S.C. 103(a) as being unpatentable over Camden (US 6,262,093, 1999) in combination with Delatour *et al.* (IDS, Therapie 1976; 31 (4); 505-515).

Camden teaches as set forth above with regard to claims 75-76, 83-97 and 99-100, a method of treating cancer by inducing apoptosis to a cell expressing abnormal p53 comprising administering a benzimidazole derivative. Camden does not teach that the benzimidazole derivative is mebendazole.

Delatour *et al.* teach the embryotoxic and antimitotic properties of benzimidazole compounds (title). Specifically, the reference discloses that in mice with Ehrlich carcinoma mebendazole inhibited tumor growth and increased survival time (abstract).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to include mebendazole as taught by Delatour *et al.* in the method taught by Camden. One would have been motivated to make these modifications because as evidenced by Delatour *et al.*, benzimidazole derivatives such as mebendazole have been shown to inhibit tumor growth. Thus, one of ordinary skill in the art would have a reasonable expectation of success that using mebendazole as taught by Delatour *et al.* in the method taught by Camden, one would achieve an additional benzimidazole derivative that induces apoptosis in cells and tumors expressing abnormal p53.

In reference to the rejection, Applicants contend that a *prima facie* case of obviousness has not been established because the prior art reference cited by the Examiner do not teach or suggest all of the claim limitation. For the reasons set forth above, Applicants contend that the Examiner has not shown that Camden teaches or suggest inhibition of cancer as the result of expression of a tumor suppressor gene and the administration of a benzimidazole. Nor does Camden teach the particular benzimidazoles found in claims 161-162 and 183 or the limitation "wherein the tumor cell is a multidrug resistant tumor cell. Moreover, Applicants argue that Delatour includes no information pertaining to induction of apoptosis as a result of administration of a benzimidazole and expression of a tumor suppressor gene. Applicants further assert that Delatour does not include any

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information pertaining to inhibition of cancer as a result of expression of a tumor suppressor and administration of a benzimidazole.

These arguments have been carefully considered, but are not found persuasive.

First, Applicants arguments pertaining to Camden have been addressed by the Examiner above and have been incorporated herein. In response to Applicants Arguments that Delatour does not include any information pertaining to induction of apoptosis, the examiner recognizes the *prima facie* case of obviousness was made because the benzimidazole derivatives disclosed by both Camden and Delatour *et al.* have close structural similarities and similar utilities. Moreover, the courts have held that "An obviousness rejection based on similarity in chemical structure and function entails the motivation of one skilled in the art to make a claimed compound, in the expectation that compounds similar in structure will have similar properties." In re Payne, 606 F.2d 303, 313, 203 USPQ 245, 254 (CCPA 1979). See In re Papesch, 315 F.2d 381, 137 USPQ 43 (CCPA 1963) (discussed in more detail below) and In re Dillon, 919 F.2d 688, 16 USPQ2d 1897 (Fed. Cir. 1991) (discussed below and in MPEP § 2144) for an extensive review of the case law pertaining to obviousness based on close structural similarity of chemical compounds. See also MPEP § 2144.08, paragraph II.A.4.(c). As such, Claims 75-77, 83-99 and 161-162 are rejected under 35 U.S.C. 103(a) as being unpatentable over Camden (US 6,262,093, 1999) in combination with Delatour *et al.* (IDS, Therapie 1976; 31 (4); 505-515).

New Rejections necessitated by amendment and upon discovery of new prior art:

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claim 9 is rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

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The phrase "the dose of benzimidazole is at least 0.05 mg/mL" in claim 9 renders the claim indefinite. The phrase "the dose of benzimidazole is at least 0.05 mg/mL" is not defined by the claim, the specification does not provide a standard for ascertaining the requisite degree, and one of ordinary skill in the art would not be reasonably apprised of the scope of the invention. In the instant case, the claim appears to be describing a dose of the benzimidazole in terms of a concentration. However, it is unclear what the actual dose will be. Identifying an amount, i.e. volume, of the dose, which will be given, may alleviate the rejection.

The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 161-162, 183 and 184 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. **THIS IS A NEW MATTER REJECTOIN.**

Amended claim 161 and claims 183-184 are drawn to a benzimidazole derivative having a particular formula with a proviso limitation setting forth that if R^3 is a H or chloro, then R^2 cannot be H if R^1 is carbamate. Applicants contend that written description support for the structural limitations can be found generally throughout the specification, such as on page 8, line 5 through page 9, line 22. Applicants further submit that they are not required to explicitly recite the exact language of the proviso in the specification. Moreover, Applicants assert that one of ordinary skill in the art, upon reading the specification, particularly the section cited herein, would have clearly recognized that Applicants contemplated the inclusion of benzimidazoles of the structure set forth in amended claims 161 (and depended claim 162 and new claims 183-184) for inclusion of the method set forth therein.

These arguments have been considered, but are not found persuasive for the reasons set forth below.

A careful review of the specification teaches the following: (1) the discovery that the

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benzimidazoles (BZs), fenbendazole (FZ) and mebendazole (MZ) can increase the expression of tumor suppressor genes and greatly augment the sensitivity of tumor cells to apoptosis induction (page 8, lines 21-22 and page 9, lines 24-26; figures 1A-B, 2); (2) the structural formula of the benzimidazoles of interest having variable R^3 groups, R^1 groups and R^2 groups (page 10, lines 16+). Thus, while it is clear that the specification teaches a generic chemical formula, there does not appear to be any recitation of the negative limitation on page 8, line 5 through page 9, line 22 or generally throughout. Furthermore, the Examiner agrees with Applicants assertion that they are not required to explicitly recite the negative, i.e. proviso, limitation. However, the specification does not appear to reasonably convey to one of skill in the art that the Applicants contemplated the inclusion of the benzimidazole derivatives having the negative limitation as recited in amended claim 161. For example, it appears from the specification that the preferred benzimidazole derivatives are mebendazole and fenbendazole. However, these two compounds do not meet the negative limitations set forth in amended claim 161. Applicant is invited to point to clear support or specific examples of the claimed limitation in the specification as-filed or remove such amendatory language in response to this action.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1-2, 12, 15-19, 21, 29, 75-76, 83, 85, and 88-96 are rejected under 35 U.S.C. 102(b) as being anticipated by Camden (US 5,880,144, 1999).

Camden teaches a method of killing lung tumor cells (A-549), breast tumor cells (MCF-7) and colon tumor cells comprising administering a benzimidazole derivative (column 6, lines 64 to 67, and column 7, Table 3). The patent further teaches a method of treating a patient having cancer comprising administering an effective amount of a benzimidazole derivative to inhibit the growth of the cancer (abstract). With regards to administration, Camden teaches (column 5, lines 1-10) that

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the benzimidazole derivatives can be administered orally, by intravenous injection, by parental administration or by injection into or around the tumor. Although Camden does not specifically teach that the administration of benzimidazole induces apoptosis, the claimed functional limitation would be an inherent property of the referenced method because as evidenced by Camden (US Patent 6,262,093, 1999), the administration of benzimidazole derivatives results in apoptosis (see column 11, line 65 to column 12, line 51). Thus, it does not appear that the claim language or limitation results in a manipulative difference in the method steps when compared to the prior art disclosure. See Bristol-Myers Squibb Company v. Ben Venue Laboratories 58 USPQ2d 1508 (CAFC 2001). Moreover, while Camden does not explicitly characterize the tumor cell lines as expressing a tumor suppressor gene such as p53, the claimed functional limitation would be an inherent property of the referenced method since the specification discusses (page 64, Table 4) that A459 tumor cells express wild-type p53. Thus, it does not appear that the claim language or limitation results in a manipulative difference in the method steps when compared to the prior art disclosure. See Bristol-Myers Squibb Company v. Ben Venue Laboratories 58 USPQ2d 1508 (CAFC 2001).

Hence, even though the claims are drawn to a mechanism by cancer cells are inhibited, the claimed method does not appear to distinguish over the prior art teaching of the same or nearly the same method. The mechanism of action does not have a bearing on the patentability of the invention if the invention was already known or obvious. Mere recognition of latent properties in the prior art does not render nonobvious an otherwise known invention. In re Wiseman, 201 USPQ 658 (CCPA 1979). Granting a patent on the discovery of an unknown but inherent function would remove from the public that which is in the public domain by virtue of its inclusion in, or obviousness from, the prior art. In re Baxter Travenol Labs, 21 USPQ2d 1281 (Fed. Cir. 1991). See M.P.E.P. 2145.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

- (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the

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subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 3, 77, 161-162 and 182-183 are rejected under 35 U.S.C. 103(a) as being unpatentable over Camden (US 5,880,144, 1999) in combination with Delatour *et al.* (IDS, Therapie 1976; 31 (4); 505-515) of record or Nasr *et al.* (Journal of Pharmaceutical Sciences 1985; 74: 831-836).

Camden teaches, as set forth above for claims 1-2, 12, 15-19, 21, 29, 75-76, 83, 85, and 88-96, a method of killing lung tumor cells (A-549), breast tumor cells (MCF-7) and colon tumor cells comprising administering a benzimidazole derivative (column 6, lines 64 to 67, and column 7, Table 3). The patent further teaches a method of treating a patient having cancer comprising administering an effective amount of a benzimidazole derivative to inhibit the growth of the cancer (abstract). With regards to administration, Camden teaches (column 5, lines 1-10) that the benzimidazole derivatives can be administered orally, by intravenous injection, by parental administration or by injection into or around the tumor.

Camden does not disclose other benzimidazole derivatives such as mebendazole.

Delatour *et al.* teach the embryotoxic and antimitotic properties of benzimidazole compounds (title). Specifically, the reference discloses that a method of inhibiting tumor growth in mice comprising administering the benzimidazole derivative, mebendazole (abstract).

Nasr *et al.* teach (page 831, paragraph bridging 1st column and 2nd) *in vivo* anticancer activity correlation of aromatic, aliphatic, and heterocyclic carbamates and their thio-isosters against both intraperitoneally implanted murine P-388 lymphocytic leukemia and L-1210 lymphoid leukemia. Specifically, the reference teaches anticancer activity of benzimidazole carbonates (page 834, Table VIII and page 835, 2nd column, 2nd full paragraph).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to combine the references so as to inhibit tumor growth because each of the benzimidazole derivatives disclosed by the references have close structural similarities and similar utilities. In the instant case, the courts have held that "An obviousness rejection based on similarity in chemical structure and function entails the motivation of one skilled in the art to make a claimed compound, in the expectation that compounds similar in structure will have similar properties." In re Payne, 606 F.2d 303, 313, 203 USPQ 245, 254 (CCPA 1979). See In re Papesch, 315 F.2d 381, 137 USPQ 43 (CCPA 1963); In re Dillon, 919 F.2d 688, 16 USPQ2d 1897 (Fed. Cir. 1991) (see in

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MPEP § 2144) for an extensive review of the case law pertaining to obviousness based on close structural similarity of chemical compounds. See also MPEP § 2144.08, paragraph II.A.4.(c). Thus, one of skill in the art would have a reasonable expectation of success that by substituting a benzimidazole derivate as taught by Delatour et al. or Nasr et al. in the method of Camden, one would achieve a method of inhibiting the growth of cancer.

Claims 23-28 and 101-106 are rejected under 35 U.S.C. 103(a) as being unpatentable over Camden (US 5,880,144, 1999) in combination with Delatour *et al.* (IDS, Therapie 1976; 31 (4); 505-515) of record or Nasr et al. (Journal of Pharmaceutical Sciences 1985; 74: 831-836) in view of Perdoma *et al.* (J. Cancer Res. Clin. Oncol. 1998, 124, 10-18) of record.

The combination of Camden and Delatour et al. or Nasr et al. teach, as set forth above with regard to claims 1-3, 12, 15-19, 21, 29, 75-77, 83, 85, 88-96, 161-162 and 182-183, a method of killing lung tumor cells, breast tumor cells and colon tumor cells comprising administering a benzimidazole derivative. The patent further teaches a method of treating a patient having cancer comprising administering an effective amount of a benzimidazole derivative to inhibit the growth of the cancer (abstract).

The combination of Camden and Delatour et al. or Nasr et al. does not teach determining the tumor suppressor status by way of Southern blotting, Northern blotting, PCR, ELISA or Western blotting (claims 23-28 and 101-106).

Perdoma *et al.* teach determining the p53 status, by Western blot analysis (page 12, 3rd paragraph) or other methods such as polymerase chain reaction (PCR), could make it possible to predict the response to therapy in certain patients (page 17, 1st column, 2nd paragraph). Perdoma *et al.* further teach that the response to cisplatin *in vivo* of NSCLC tumor lines was dependent on p53 status (page 17, 1st column, 2nd paragraph). Specifically, the reference teaches wt-p53 tumors showed a regression in size of around 60%, whereas mt-p53 tumors stopped growing (page 17, 1st column, 2nd paragraph).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to determine the status of a tumor suppressor gene, like p53, in a tumor cell prior to administering a benzimidazole derivative using techniques such as Western blot, PCR or other methods of analysis. One would have been motivated to do so because as taught by Perdoma

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et al., analysis of p53 status, by immunohistochemical or other methods such as the polymerase chain reaction (PCR), could make it possible to predict the response to therapy in certain patients (page 17, 1st column, 2nd paragraph). Thus, one of skill in the art would have a reasonable expectation of success that by measuring the status of the tumor suppressor gene in view of Perdoma, one would achieve an effective method of predicting the outcome of benzimidazole therapy.

Claims 13-14 and 86-87 are rejected under 35 U.S.C. 103(a) as being unpatentable over Camden (US 5,880,144, 1999) in combination with Delatour *et al.* (IDS, Therapie 1976; 31 (4); 505-515) of record or Nasr *et al.* (Journal of Pharmaceutical Sciences 1985; 74: 831-836) in view of Lucci *et al.* (Cancer; 86:300-311, published online on November 2000).

The combination of Camden and Delatour *et al.* or Nasr *et al.* teach, as set forth above with regard to claims 1-3, 12, 15-19, 21, 29, 75-77, 83, 85, 88-96, 161-162 and 182-183, a method of killing lung tumor cells, breast tumor cells and colon tumor cells comprising administering a benzimidazole derivative. The patent further teaches a method of treating a patient having cancer comprising administering an effective amount of a benzimidazole derivative to inhibit the growth of the cancer (abstract).

The combination of Camden and Delatour *et al.* or Nasr *et al.* does not teach that the tumor cell is a multidrug resistant tumor cell, wherein the tumor cell is a breast tumor cell.

Lucci *et al.* teach multidrug resistance modulators and doxorubicin synergize to elevate ceramide levels and elicit apoptosis in drug-resistant cancer cells, specifically drug resistant human breast cancer cells lines. Moreover, the reference teaches that multidrug resistance is a formidable roadblock to the effective treatment of cancer by conventional chemotherapy, wherein the resistance complicates treatment in many instances (page 300, 1st paragraph).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to use a multidrug resistant cell line, such as a breast cancer cell, in the method taught by Camden in view of the teachings of Lucci *et al.* One would have been motivated to do so because as taught by Lucci, multidrug resistance is a formidable roadblock to the effective treatment of cancer by conventional chemotherapy, wherein the resistance complicates treatment in many instances. Thus, one of ordinary skill in the art would have a reasonable expectation of success that by administering a benzimidazole derivative to multidrug resistant cell, one would achieve a method

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of inhibiting tumor growth in a patient that has already become resistant to conventional chemotherapy.

Note: Claims 10, 20 and 98 are objected to as being dependent from a rejected independent claim.

All other rejections and/or objections are withdrawn in view of applicant's amendments and arguments there to.


Any inquiry concerning this communication or earlier communications from the examiner should be directed to Brandon J. Fetterolf, PhD whose telephone number is (571)-272-2919. The examiner can normally be reached on Monday through Friday from 8:30 to 5:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Jeff Siew can be reached on 571-272-0787. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Brandon J Fetterolf, PhD
Examiner
Art Unit 1642

BF


JEFFREY SIEW
SUPERVISORY PATENT EXAMINER
11/14/05

Notice of References Cited	Application/Control No. 10/043,877		Applicant(s)/Patent Under Reexamination MUKHOPADHYAY ET AL.	
	Examiner Brandon J. Fetterolf, PhD		Art Unit 1642	Page 1 of 2

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-5,880,144	03-1999	Camden, James Berger	514/397
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Freshney (Culture of Animal Cells, A Manual of Basic Technique, Alan R. Liss, Inc., 1983, New York, p4)
	V	Dermer (Bio/Technology, 1994, 12:320)
*	W	Delatour et al. (IDS, Therapie 1976; 31 (4); 505-515)
	X	Nasr et al. (Journal of Pharmaceutical Sciences 1985; 74: 831-836)

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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	Examiner Brandon J. Fetterolf, PhD		Art Unit 1642	Page 2 of 2

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	D	US-			
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	K	US-			
	L	US-			
	M	US-			

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	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
*	U	Perdoma et al. (J. Cancer Res. Clin. Oncol. 1998, 124, 10-18)
	V	Lucci et al. (Cancer; 86:300-311, published online on November 2000)
	W	Su et al. (PNAS 1998; 95: 14400-14405)
	X	

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EXHIBIT 5

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Tapas Mukhopadhyay, *et al.*

Serial No.: 10/043,877

Filed: January 9, 2002

For: ANTIHELMINTHIC DRUGS AS A
TREATMENT FOR
HYPERPROLIFERATIVE DISEASES

Group Art Unit: 1642

Examiner: B. J. Fetterolf

Atty. Dkt. No.: INRP:095US

**THIRD DECLARATION OF TAPAS MUKHOPADHYAY, SUNIL CHADA,
ABNER MHASHILKAR, AND JACK A. ROTH UNDER 37 C.F.R. §1.131**

We, Tapas Mukhopadhyay, Sunil Chada, Abner Mhashilkar, and Jack A. Roth, hereby declare as follows:

1. We are the joint inventors of the subject matter claimed in the above-referenced patent application, U.S.S.N. 10/043,887, filed January 9, 2002.

2. We previously submitted a declaration to set forth facts demonstrating that we both conceived the idea of the invention as reflected in the claims of the above-referenced patent application and determined that it functioned, prior to March 9, 1999.

3. In the present declaration we are submitting facts demonstrating that we steadily progressed in our research to confirm that our invention functioned in an animal model, which was in accordance with our initial understanding. We continually and

diligently conducted these studies from the time we conceived of our invention until the time our U.S. Provisional patent application, U.S.S.N. 60/261,346, was filed on January 11, 2001. Evidence of our diligence is set forth in Exhibits 1-3, discussed in detail below.

4. Submitted as Exhibit 1 to this declaration is a copy of a draft manuscript of our experiments and results, entitled "Potent Induction of Apoptosis by Anthelmintics in Human Lung Cancer Cells: Involvement of Wild-Type p53 and p21 Kinase Inhibitor." The studies set forth in this manuscript and the preparation of this manuscript took place prior to January 14, 2000.

5. Submitted as Exhibit 2 to this declaration is a copy a series of experiments and results involving the use of benzimidazoles in the treatment of p53 wild type expressing tumor cells, ending with animal models, as evidenced by the laboratory notebook of Dr. Jiichiro Sasaki, who worked under the direction of Dr. Tapas Mukhopadhyay. These experiments took place between November 10, 2000 and September 9, 2001.

6. Submitted as Exhibit 3 to this declaration is a copy of a draft manuscript of our experiments and results, including in animal models, entitled "Mebendazole: A Novel Microtubule Agent Having Potent Antitumor Activity," which was submitted for publication on October 25, 2001.

7. Exhibit 2 shows the preparation and results of a series of experiments pertaining to the benzimidazole drug, mebendazole (labeled MZ) for the treatment of cancer. Experiments pertaining to the treatment of cancer cells with mebendazole are listed on the following dates: November 11, 2000; November 15, 2000; December 5, 2000; December 8, 2000; December 12, 2000; January 11, 2001; January 12, 2001; January 18, 2001; February 10, 2001; February 20, 2001; February 23, 2001; February 26, 2001; March 2-6, 2001; March 19, 2001; March 20, 2001; March 29-31, 2001; April 3-4, 2001, April 14, 2001; April 19, 2001; April 26, 2001; June 6, 2001, June 7, 2001; June 16, 2001; July 24, 2001; July 26-28, 2001; August 1, 2001; August 4, 2001; August 5, 2001; August 7, 2001; August 31, 2001; August 31, 2001 and September 9, 2001. Of note, the last two entries pertain to the use of mebendazole in animal models.

8. Exhibit 3 shows the draft manuscript pertaining to the treatment of cancer cells with mebendazole on the inhibition of human tumor xenografts in mice. See Exhibit 3, Abstract, page 2. In accordance with our in vitro data, our animal model data showed that Mebendazole inhibited lung cancer growth. For example oral administration of mebendazole to mice previously injected with A549 lung cancer cells resulted in an 80% reduction in tumor count as compared to the control. See Exhibit 3, Results, page 10-11.

9. All work disclosed in the invention disclosure form was conducted in the United States of America.

10. Therefore, the invention as reflected in claims 75-77, 83-106, 161-162 and 184 of the above-referenced patent application was conceived of prior to March 9, 1999 and diligently reduced to practice.

11. We hereby declare that all statements made by our own knowledge are true and all statements made on information and belief are believed to be true and further that statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment under § 100 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

4/25/2006
Date

Date

Date

Date

Tapas Mukhopadhyay
Tapas Mukhopadhyay

Sunil Chada

Abner Mhashilkar

Jack A. Roth

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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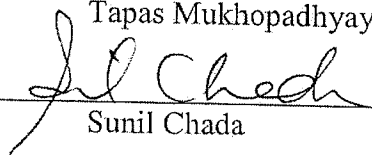
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Date
04/20/06

Date

Tapas Mukhopadhyay


Sunil Chada

Date

Abner Mhashilkar

Date

Jack A. Roth

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Date

Tapas Mukhopadhyay

Date

Sunil Chada

4/18/06

Date

Abner Mhashilkar

Date

Jack A. Roth

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Tapas Mukhopadhyay, *et al.*

Serial No.: 10/043,877

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diligently conducted these studies from the time we conceived of our invention until the time our U.S. Provisional patent application, U.S.S.N. 60/261,346, was filed on January 11, 2001. Evidence of our diligence is set forth in Exhibits 1-3, discussed in detail below.

4. Submitted as Exhibit 1 to this declaration is a copy of a draft manuscript of our experiments and results, entitled "Potent Induction of Apoptosis by Anthelmintics in Human Lung Cancer Cells: Involvement of Wild-Type p53 and p21 Kinase Inhibitor." The studies set forth in this manuscript and the preparation of this manuscript took place prior to January 14, 2000.

5. Submitted as Exhibit 2 to this declaration is a copy a series of experiments and results involving the use of benzimidazoles in the treatment of p53 wild type expressing tumor cells, ending with animal models, as evidenced by the laboratory notebook of Dr. Jiichiro Sasaki, who worked under the direction of Dr. Tapas Mukhopadhyay. These experiments took place between November 10, 2000 and September 9, 2001.

6. Submitted as Exhibit 3 to this declaration is a copy of a draft manuscript of our experiments and results, including in animal models, entitled "Mebendazole: A Novel Microtubule Agent Having Potent Antitumor Activity," which was submitted for publication on October 25, 2001.

7. Exhibit 2 shows the preparation and results of a series of experiments pertaining to the benzimidazole drug, mebendazole (labeled MZ) for the treatment of cancer. Experiments pertaining to the treatment of cancer cells with mebendazole are listed on the following dates: November 11, 2000; November 15, 2000; December 5, 2000; December 8, 2000; December 12, 2000; January 11, 2001; January 12, 2001; January 18, 2001; February 10, 2001; February 20, 2001; February 23, 2001; February 26, 2001; March 2-6, 2001; March 19, 2001; March 20, 2001; March 29-31, 2001; April 3-4, 2001, April 14, 2001; April 19, 2001; April 26, 2001; June 6, 2001, June 7, 2001; June 16, 2001; July 24, 2001; July 26-28, 2001; August 1, 2001; August 4, 2001; August 5, 2001; August 7, 2001; August 31, 2001; August 31, 2001 and September 9, 2001. Of note, the last two entries pertain to the use of mebendazole in animal models.

8. Exhibit 3 shows the draft manuscript pertaining to the treatment of cancer cells with mebendazole on the inhibition of human tumor xenografts in mice. See Exhibit 3, Abstract, page 2. In accordance with our in vitro data, our animal model data showed that Mebendazole inhibited lung cancer growth. For example oral administration of mebendazole to mice previously injected with A549 lung cancer cells resulted in an 80% reduction in tumor count as compared to the control. See Exhibit 3, Results, page 10-11.

9. All work disclosed in the invention disclosure form was conducted in the United States of America.

10. Therefore, the invention as reflected in claims 75-77, 83-106, 161-162 and 184 of the above-referenced patent application was conceived of prior to March 9, 1999 and diligently reduced to practice.

11. We hereby declare that all statements made by our own knowledge are true and all statements made on information and belief are believed to be true and further that statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment under § 100 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

Date

Tapas Mukhopadhyay

Date

Sunil Chada

Date

Abner Mhashilkar

4/19/06

Date



Jack A. Roth

EXHIBIT 1

**Potent induction of apoptosis by anthelmintics in human lung cancer cells:
involvement of wild-type p53 and p21 kinase inhibitor¹**

Tapas Mukhopadhyay² and Jack A. Roth

Section of Thoracic Molecular Oncology, Departments of Thoracic and Cardiovascular Surgery
[TM, JAR] and Tumor Biology [JAR], The University of Texas M. D. Anderson Cancer Center,
Box 109, 1515 Holcombe Blvd., Houston, TX 77030.

Running Title: Bendimidazole-induced apoptosis

¹This study was partially supported by grants from Specialized Program of Research Excellence (SPORE) in Lung Cancer (P50-CA70907); by gifts to the Division of Surgery and Anesthesiology from Tenneco and Exxon for the Core Laboratory Facility; by The University of Texas M. D. Anderson Cancer Center Support Core Grant (CA16672); and by a sponsored research agreement with Introgen Therapeutics, Inc.

²To whom correspondence and request for reprints should be addressed at the Department of Thoracic and Cardiovascular Surgery, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 23, Houston, TX 77030

Tel: (713) 745-4542; Fax: (713) 794-4669; E-Mail: tmukhopa@notes.mdacc.tmc.edu

The abbreviations used are: MZ, (methyl 5-benzoylbenzimidazole-2-carbamate; FZ, methyl 5-(phenylthio)-2-benzimidazole carbamate (fenbendazole); TUNEL, terminal-deoxynucleotidyltransferase (TnT)- mediated dUTP-biotin nick end labeling; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; Hoechst 33342, benzidine; NSCLC, non-small cell lung cancer; DMSO, dimethylsulfoxide; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; MOPS, morpholinepropanesulfonic acid; PI, propidium iodide.

Summary

We have studied the effect of the broad-spectrum anthelmintic benzimidazoles on the regulation of apoptosis in the human lung cancer cell lines. In this study, the in vitro effect of the benzimidazole compounds fenbendazole and mebendazole, on human lung cancer cell lines was determined. These drugs dramatically inhibited the growth of lung cancer cells in culture. Western blot analysis done using specific antibodies against Bcl-2, Bcl-xl, Bax, RB, cdc2, Cdk2, cyclin A, cyclin D, and p53 showed that treatment with fenbendazole and mebendazole did not alter the levels of any of these proteins except p53. The drug treatment also induced a dose- and time-dependent nuclear accumulation of wild-type p53 whose kinetics correlated well with the induction of apoptotic cell death. The effect of these benzimidazoles was further assessed in a number of human cell lines. Interestingly, only cell lines containing the wild-type p53 were highly sensitive to growth inhibition and apoptosis after benzimidazole treatment. The presence of wild-type p53 correlated well with enhanced growth arrest, micronucleation, and p53-dependent apoptosis in drug-treated cells. In addition, p53, MDM2 and p21^{Cip1/WAF1} protein levels significantly increased by 24h after drug treatment. However, cell lines carrying mutated p53 were quite resistant to the cytotoxic effect of the drugs. Restoration of wild-type p53 function made tumor cells more sensitive to FZ and MZ induced apoptosis. The ability of benzimidazole to induce apoptosis in HeLa and SiHa cell lines, which express HPV-E6 protein as a dominant negative factor for p53-mediated cell death, was diminished. Thus, our collective findings strongly suggest that a p53-dependent mechanism contributes to the cytotoxicity induced by benzimidazoles in human cancer cells.

Benzimidazoles are broad-spectrum anthelmintics that display excellent activity against parasitic nematodes and, to a lesser extent, cestodes and trematodes (1). Benzimidazoles are effective antiprotozoal agents and have antifungal activity (2). It is currently believed that benzimidazoles exert their cytotoxic effects by binding to the microtubule system and disrupting its functions (3)(4). The suggestion that tubulin is a target for benzimidazoles has been supported by the results of drug-binding studies using enriched extracts of helminth and mammalian tubulin (3). Moreover, competitive drug-binding studies using mammalian tubulin have shown that benzimidazoles compete for colchicine binding and inhibit the growth of L1210 tumor cells in vitro (5)(3). However, benzimidazoles are selectively toxic to nematodes when administered as anthelmintics but are not toxic to the host (1). In contrast, benzimidazoles suppress the in vitro polymerization of mammalian tubulin (2). Differences in the affinity between host and parasite macromolecules for benzimidazole (6)(7) as well as the pharmacokinetics of benzimidazoles between the host and the parasite have been suggested as the factors responsible for the selective toxicity of benzimidazoles (8) but the actual molecular basis of this selective toxicity remains unclear.

Of all the proteins whose loss of function is associated with cancer development, p53 is the best known. In its wild-type form, it may function as a critical regulator of genotoxic stress and apoptosis (9)(10). Studies of the wild-type protein have shown that DNA damage or oxidative stress can increase the cellular accumulation of this protein by increasing its stability in stressed cells (10)(11)(12). Increases in the level of the p53 protein may in turn directly facilitate DNA repair, or they may indirectly inhibit cell-cycle progression or induce apoptosis (10)(12). Conversely, loss of p53 function may allow damaged cells to survive and permit DNA damage to accumulate, further promoting cellular transformation (11)(13)(14)(15). Therefore, genotoxic stress surveillance and concomitant p53 accumulation are important primary processes in damaged cells. In the study described here, we exposed a number of human lung cancer cell

lines of differing p53 status to benzimidazoles to evaluate the cytotoxicity of the drugs and found that the frequency of apoptosis was greater in the benzimidazoles-exposed cells. We also correlated the involvement of the p53 gene with the degree of drug sensitization and found that cell lines containing a functional p53 gene were more sensitive to benzimidazoles.

Benzimidazoles drugs are commercially available and show remarkable safety when used as anthelmintics in the treatment of many veterinary and human helminthiases. It is therefore possible that these drugs could be used clinically to inhibit the growth cancer cells. Our results demonstrate that BZs rapidly induces the production of the wild-type p53 protein in human lung cancer cell lines and concomitantly induces apoptosis.

Experimental Procedures

Materials-Methyl-5-benzoylbenzimidazole-2-carbamate (mebendazole [MZ]) and methyl-5-(phenylthio)-2-benzimidazolecarbamate (fenbendazole [FZ]) were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were purchased from Sigma unless otherwise indicated.

Cell Culture-Non-small cell lung cancer (NSCLC) cell lines were used in our studies. All except A549 were gifts from Drs. Adi Gazdar and John Minna (The University of Texas Southwestern Medical Center, Dallas, TX). All other cell lines were obtained from the American Type Culture Collection (Rockville, MD). In all cases, cell lines were grown according to the directions provided by suppliers. All culture media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) and antibiotics (100 mg/ml of streptomycin and 100 IU/ml of penicillin BRL). Mebendazole and fenbendazole were dissolved in dimethylsulfoxide (DMSO) and then diluted in phosphate-buffered saline (PBS) (1:1). When reagents containing DMSO were used, an equal volume of DMSO was added to the control cells.

In vitro cell culture and proliferation assay-All cell lines were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum and 5% CO₂. All experiments were done when the cells were 70% confluent. For cell growth measurements, 5×10^4 cells were plated in each well of six-well plates. Control cells and cells treated with benzimidazoles (0.05 μ g/ml) were trypsinized and counted using a hemocytometer. Experiments were done in triplicate, and the mean and standard deviation were determined by standard methods. Using the Curve Fit 1.3 program, the 50% growth inhibitory concentrations (IC₅₀) were extrapolated from a plot of the percent of control cell growth (triplicate determinations) versus drug concentration after 24 h of treatment.

RNA Isolation and Northern Blot Analysis-Total RNA was isolated from subconfluent cultures using the guanidinium thiocyanate method (3). After this, 20 μ g of the RNA was electrophoresed in a denaturing 1.2% agarose/morpholinepropanesulfonic acid (MOPS)-

formaldehyde gel, transferred onto a nitrocellulose membrane, and hybridized to ^{32}P -radiolabeled p53 cDNA probes, as described elsewhere (16). The ^{32}P -labeled probes were generated using random primers ($>8 \times 10^8$ cpm/ μg). Blots were washed at 65°C in $2\times$ standard saline citrate (SSC) for 30 min and then washed twice at 60°C in 0.1% sodium dodecyl sulfate (SDS) and 0.1X SSC. The cDNA probes used were 1.2-kb human p53 cDNA and an 800-bp fragment of human p21 cDNA.

Antibodies-The following antibodies were used: mouse anti-p21 monoclonal antibody WAF-1(Ab-1) Oncogene Sciences (Cambridge, MA); mouse monoclonal anti-Cyclin A (Sigma St Louis, MO), rabbit antihuman cyclin D (Upstate Biotech. Inc.) mouse anti-RB monoclonal antibody (Pharmingen, San Diego, CA), and mouse anti-c-myc monoclonal antibody (Invitrogen, Carlsbad, CA). Mouse anti-BCL-2 (100) monoclonal antibody, rabbit anti-Bcl-xl (S-19) polyclonal antibody, rabbit anti-Bax (N-20) polyclonal antibody, mouse anti-MDM2 (SM P14) monoclonal antibody, mouse anti-2 (100) monoclonal antibody, mouse anti-p53 (Bp53-12) monoclonal antibody, mouse anti-Cdk-2 (M2-G) goat polyclonal, antibody, and mouse anti-Cdc-2 p34 monoclonal antibody were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Amersham International (Arlington Heights, IL).

Nuclear Staining Assay- Cells were seeded onto chamber slides and treated with various reagents, after which cell monolayers were washed twice with ice-cold PBS (pH 7.4). Thereafter, cells were fixed for 5 min at 20°C in 10% formalin. The PBS washing step was then repeated once. To stain the nuclei, the cells were incubated for 10 min with 10 $\mu\text{g}/\text{ml}$ of Hoechst 33342 and then washed with PBS. Coverslips seeded with the stained cells were mounted in 80% glycerol in PBS containing 1 mg/ml P-phenylenediamine and examined with a Nikon epifluorescence microscope.

Apoptotic assay and TdT FACS analysis-Apoptotic assay was done using M30 CytoDEATH

apoptotic cell death assay kit (Boehringer Mannheim). Cells were grown on chamber slides, control and benzimidazole treated cells were stained with Mouse monoclonal antibody(clone M30) as per manufacturer's instructions. Apoptotic cells were examined with a Nikon microscope and photomicrographed. For TdT FACS analysis control and treated cells were collected by trypsinization, washed in PBS, and fixed overnight in 70% ethanol. The next day, cells were rehydrated in PBS for 30 min, centrifuged, and resuspended in PBS. For DNA analysis, propidium iodide (PI) was added at 50 $\mu\text{g/ml}$, and the cells were incubated in the presence of RNase A (15 mg/ml for 30 min at 37°C). To detect DNA strand breaks associated with apoptosis, cells were fixed in 1% formaldehyde for 15 min at 4°C, rinsed in PBS, and stored at 4°C in ice-cold 70% ethanol. Before staining, the cells were washed in PBS, and 10^6 cells were resuspended in 50 ml of cacodylated buffer containing 100 $\mu\text{g/ml}$ TdT enzyme and 0.5 nM biotin-16 dUTP for 30 min at 37°C. Cells were washed in PBS and resuspended in 100 ml of 4X SSC containing 2.5 mg/ml fluoresceinated avidin, 0.1% Triton X-100, and 5% dry fat milk and then incubated at room temperature for 30 min in the dark. Finally, cells were washed in PBS and resuspended in PI buffer. Flow cytometry was carried out in a fluorescence-activated cell sorter (Epics Elite; Coulter, Inc., Hialeah, FL).

Immunohistochemical Staining-Cells were seeded onto glass coverslips and fixed as described above. The cells were blocked at 37°C for 30 min with 2% bovine serum albumin, 5% fetal bovine serum, and 5% normal goat serum in PBS. The cells were then incubated at room temperature for 45 min with anti-p53 (Ab-2) antibody (1:1000 dilution) in blocking buffer and washed with PBS. The cells were then incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (Amersham). After washing, the immunocomplex was detected by an avidin-biotin complex kit, and slides were mounted as described above.

Cell Lysates and Immunoblotting-Cells were grown in 6-cm dishes, cultured, and treated as described above. To prepare the whole-cell lysates, the medium was removed. Then, cells were

washed twice with ice-cold Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris; pH 7.6) and lysed with 0.5 ml of lysis buffer (10 mM Tris, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM benzamidine, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mM sodium orthovanadate) for 15 min. The lysed cells were then transferred to 1.5-ml microtubes and centrifuged at $15,000 \times g$ for 10 min at 4°C. Then, the supernatants were collected, mixed with Laemmli's sample buffer, and subjected to western blot analysis as described elsewhere (5). Blots were probed with anti-p53 monoclonal antibody B p53-12 (Santa Cruz Biotechnology, Inc.), and immunocomplexes were detected using an Enhanced Chemiluminescence kit (Amersham) according to the manufacturer's directions. Blots were then reprobed, this time with anti-actin monoclonal antibody (Amersham), to show that protein loading was equal. After that, blots were again reprobed, this time with anti-WAF monoclonal antibody (Pharmingen, San Diego, CA).

Pulse-Chase Experiments-Cells were treated with 0.05 µg/ml fenbendazole or mebendazole for 24 h. After this, control (mock-treated) and drug-treated H460 cells were incubated with 10 µg/ml cycloheximide in drug-free medium for different times and then processed to obtain total cell extracts. Finally, samples were denatured by boiling them in SDS-loading buffer (100 mM Tris, pH 6.8; 2% SDS; 0.1% bromphenol blue; 10% glycerol; 25 µM β-mercaptoethanol) and loaded onto a 10% SDS-polyacrylamide gel. Blots were probed with anti-p53 monoclonal antibody B p53-12 (Santa Cruz Biotechnology, Inc.) and anti-actin monoclonal antibody (Amersham). The immunocomplexes were detected using the Enhanced Chemiluminescence kit according to the manufacturer's directions as described above. The intensities of the bands were quantitated with a PhosphorImager using ImageQuant™ Software (Molecular Dynamics, Sunnyvale, CA).

Results

BZ-induced Apoptosis in Human NSCLC Cell Line H460-We tested the cytotoxic effect of fenbendazole and mebendazole on the human NSCLC cell line H460. After treatment with fenbendazole (0.05 $\mu\text{g/ml}$) or mebendazole (0.05 $\mu\text{g/ml}$) for 48 h, the cells became rounded and loosely attached to the plates, suggesting the cells were viability. Further analyses of the cell samples showed that the cells were undergoing apoptosis. Most of the morphological hallmarks associated with apoptosis were detectable, including cell shrinkage, DNA fragmentation, and chromatin condensation (Fig. 1A). The DNA strand breaks typical of the apoptosis, were also demonstrated by TUNEL and benzimidine staining (Fig. 1, A and B). The morphological changes produced by FZ and MZ treatments were indistinguishable. Gross morphological changes associated with a loss of viability were observed by in 24h after treatment, whereas signs of apoptosis, detected by DNA staining and TUNEL assays, became apparent by 24 h after drug treatment. Because of the close structural resemblance between FZ and MZ (i.e., both have the benzamidazole core structure), it is not surprising that they both induced apoptosis and the nuclear accumulation of p53 (Fig. 1C).

Induction of Nuclear Accumulation of p53-Both fenbendazole and mebendazole showed an apoptotic effect on the H460 cells. To rule out the possibility that fenbendazole and mebendazole were affecting other proteins known to play a role in various apoptotic pathways, we evaluated the effect of these drugs on a panel of such proteins. Many of these proteins are known activators or suppressors (e.g., Bcl-2, Bax, RB) of apoptosis and have already been shown to be expressed in these cells. Specifically, the levels of these proteins were evaluated by western blot analyses following fenbendazole and mebendazole treatments. Although the levels of nuclear p53, p21, and MDM2 were enhanced, there were, interestingly, no changes in the levels of Bcl-2, cyclin A, cyclin D, Cdc 2, Bcl-xl, Bax, RB, or Cdk-2 (Fig. 2). The protein level of the phosphorylated form of RB also remained unchanged after treatment for 24 h (Fig. 2).

Because fenbendazole and mebendazole specifically increased the amount of nuclear p53 detectable in the 460 cell line (Fig. 3), the apoptotic effect of these drugs on these NSCLC cells was presumably p53 dependent. There was also a positive correlation between the ability of fenbendazole and mebendazole to induce apoptosis and the ability of these agents to mediate the nuclear accumulation of p53. As a result of p53 protein accumulation, the p53-regulated genes were also expressed at much higher levels (Fig. 3). MDM2 and p21 levels analyzed by western blot analysis after fenbendazole treatment increased.

Kinetics of Induction of p53 and Apoptosis-The effect of fenbendazole and mebendazole on the nuclear accumulation of p53 appeared to be gradual and was not significant within the first hour of exposure. However, it became significantly detectable at about 16 h and peaked at 24 h (Fig. 4A). The appearance of p53 in response to drug treatment also coincided with the initiation of apoptosis, which was detectable by 24 h. Northern blot analysis, however, revealed no significant changes in the p53 mRNA levels (Fig. 4B), indicating that the nuclear accumulation of p53 was not due to an increase in p53 transcripts. As evidence that the p53 induced in these cells was functional, the level of the transcript for one of the p53 target genes, p21/WAF1, was significantly increased at the time the p53 level peaked (Fig. 4B).

The induction of p53 became detectable at a dose of 0.01 $\mu\text{g/ml}$ fenbendazole (Fig. 5A). However, the induction appeared to occur abruptly, which suggests the presence of a cooperative mechanism. Similar observations were made under serum-free conditions, suggesting that the source of the cooperative effect was not factors in the serum (data not shown). As predicted in light of the northern blot experiment, the steady-state p21 level (in parallel with the p53 protein level) did increase significantly over that in the control experiment in the H460 cells but not in the mutant p53-carrying H322 cells. The results of the DNA fragmentation assay, which assessed the effect of fenbendazole in inducing of apoptosis, and the assays of nuclear accumulation of p53 correlated well (Fig. 5B).

Increased Half-life of p53 in Drug-treated Cells-Because fenbendazole and mebendazole did not seem to affect the transcriptional rate of the p53 gene, we evaluated the effect of these agents on the stability of p53 in H460 cells and noted that both agents were able to prolong the half-life of the p53 protein in H460 cells. Because the results were similar for fenbendazole and mebendazole treatments, only data obtained from fenbendazole-treated cells are presented. Specifically, the half-life of the wild-type p53 in untreated H460 cells was about 6-8 h compared to 24 h in the drug-treated cells (Fig. 6). The former finding is consistent with the previously reported half-life of p53 in a number of cancer cell lines, which far outstripped the 20-to 30-min half life seen in normal fibroblasts (17).

Selective Induction of Apoptosis by Fenbendazole and Mebendazole in Tumor Cells

Carrying Wild-type p53-Studies to determine the IC_{50} for fenbendazole and mebendazole were performed on a panel of six different human cancer cell lines: two carrying the wild-type p53 gene and actively expressing the p53 protein, (H460 and A549); one marked by homozygous deletion of the p53 gene and lacking p53 gene expression (H358); two expressing mutant p53 (H322 and H596); and one in which wild-type p53 is inactivated by human papilloma virus E6 protein. Comparison of the concentrations of bendimidazoles necessary to inhibit the growth of the different cell lines by 50% (IC_{50}) indicated that wild-type p53 containing cell lines were 2-to 7-fold more sensitive than the p53-mutated or deleted cell lines (Table I). A dose of 166 nM (~0.05 mg/ml) was chosen for these studies because it had been shown previously that this concentration was sufficient to induce wild-type p53 after 24 h of treatment.

To further investigate the relationship between the induction of functional p53 and the subsequent apoptosis mediated by these drugs, we posited that if BZs require wild-type p53 in order to exert their effect, then fenbendazole and mebendazole would not be cytotoxic to the many tumor lines producing a mutated p53 protein. To investigate this possibility, human cell lines derived from NSCLC origins of differing p53 status were further analyzed by a 5-day cell

growth assay. Results for three cell lines (H460, H322, H1299) are shown in Fig. 7. H322 is a human lung adenocarcinoma line that produces a mutant p53 protein. The mutant p53 protein is generally more stable (18)(19) and this was reflected in the present case by the presence of higher amounts of the p53 protein in the nucleus of H322 cells prior to drug treatment (Fig. 7). H1299 is a p53 gene-deleted human NSCLC cell line and does not express any p53 protein. However, fenbendazole and mebendazole induced nuclear accumulation of p53 only in the H460 cell line, which carries wild-type p53 genes. Fenbendazole and mebendazole therefore appeared to be significantly effective in killing wild-type p53-containing cancer cells.

An analysis of 18 human tumor cell lines (Table II) was performed, which showed that these drugs had an effect on nuclear accumulation of p53 only in the cell lines carrying the wild-type p53 gene. Both drugs induced some degree of growth inhibition; rather than apoptosis, in cell lines that contained mutated or deleted p53; however, they induced greater growth inhibition in cell lines containing wild-type p53: As expected, HeLa and SiHa cervical cancer cell lines containing wild-type p53 along with HPV-E6 protein were less sensitive to the inductive effect of these drugs on apoptosis and p53 accumulation.

In order to further confirm that bendamidazoles works through p53 mediated pathway the synergistic effect of Ad5p53 and Fenbendazole on tumor cell growth was examined in four human lung cancer cell lines that differed in p53 status but were all transduced with Ad5p53: H1299 (p53 deleted), H322 (p53 mutated), H460 (wt p53) and A549 (wt p53). Because our initial dose-response studies indicated that 0.05 ug/ml FZ induced high levels of wt p53 protein expression in H460 cells without toxicity, we used this concentration for all our proliferation assays. In those assays, growing, cultured cells were trypsinized and plated (10^4 cells/well) and then infected the next day with Ad5p53 at 1 MOI. Viral supernatant was then added, after which cells were incubated for 24 h, washed with PBS, fed fresh medium or incubated with medium containing FZ for 24 h, washed again, and fed fresh medium. In contrast, uninfected cells

(controls) were treated with FZ for 24 h, washed with PBS, fed fresh medium, and then subjected to a 3-day growth assay (Fig.8A).

Our experimental results indicated that d1312 (empty vector) had no effect in combination with FZ (Fig. 8B). When the four lung cancer cell lines were transiently infected with 1 MOI Ad5p53 for 24 h, no growth suppression was observed, regardless of p53 status. When all four lines were treated with FZ alone for 24 h, the p53-mutated and deleted cells were not growth inhibited, whereas the wt p53 H460 cells was significantly so. However, a striking growth inhibition was observed in all four cell lines when the Ad5p53-transduced cells (1 MOI, 24 h) were treated with FZ for 24 h and were grown in normal medium for a 3-days growth assay. Our findings suggest that transducing of Adp53 will induce efficient p53-mediated killing of tumor cells in the presence of p53 superinduction by FZ. The A549 cells (containing wt p53) showed 11% apoptotic cells following FZ treatment. Low dose wild-type Adp53 had no apoptotic effect, whereas the A549 cells showed 30% apoptotic cells death 48 h after combination treatment, as shown by terminal deoxynucleotidyl transferase (TdT) staining analysis via fluorescence-activated cell sorting (FACS) as shown in Fig. 8C. These results suggest that FZ works through a p53 dependent pathway.

Inhibition of apoptotic effect by the dominant negative factor E6-To provide more direct evidence that the cytotoxic effect of fenbendazole and mebendazole correlates with the availability of functional p53 in the cell, we studied HeLa and SiHa ovarian cancer cells line that produce the E6 protein, which acts as a dominant negative factor for wild-type p53. This production of E6 is attributable, at least in part, to a dominant negative mechanism involving the degradation of the endogenous wild-type molecule. In this experiment, the HeLa (HPV-18) and SiHa (HPV-16) cells were treated with fenbendazole for 48 h, and the total cell proteins were subjected to immunoblot analysis for p53 protein production. However, no induction of p53 production was observed (**Fig. 9**), suggesting that the E6-mediated degradation of p53 was

utilizing a path different from that used in normal cells. Because these results could also have been due to a decrease in the MDM2 protein level after fenbendazole treatment, we reprobed the membrane with MDM2 monoclonal antibody. However, MDM2 protein production remained low in both control and fenbendazole-treated HeLa and SiHa cells, whereas MDM2 production in the H460 cells increased several times with the increase in the p53 protein levels. Further, in these dominant negative lines, the level of the p21 protein did not increase above the base level after treatment with these drugs. This clearly showed that fenbendazole-mediated cell killing had become less effective in cells producing the wild-type p53-inactivating E6 protein, thereby limiting the ability of fenbendazole and mebendazole to mediate apoptosis. This finding may, in part, explain why killed those cells containing wild-type p53. However, one cannot rule out the possibility that a pathway other than the p53 pathway is also involved in the cytotoxic effect of these drugs.

Discussion

We observed that the BZs fenbendazole and mebendazole selectively induced apoptosis in human NSCLC cells. Moreover, several lines of evidence in our study suggested that this apoptotic effect was mediated, at least in part, by the p53 protein. First, the kinetics of the induction of apoptosis and p53 accumulation were similar. Second, these compounds failed to induce apoptosis in mutant p53 cell lines and were also ineffective in inducing p53 production. Third, the production of an E6 protein acting as a dominant negative factor in the production of the wild-type p53 protein was sufficient to counteract the cytotoxic effect of these drugs. Finally restoration of wild-type p53 function by an adenoviral vector made tumor cells more sensitive to FZ and MZ induced apoptosis.

It is widely believed that restoring or enhancing wild-type p53 functions in tumor cells may one day be used to successfully treat many human cancers (20). Certainly, the *p53* gene is the most commonly mutated gene in human cancer (21)(22), and the resultant mutation of the p53 protein often inactivates tumor suppressor function, even though 40-50% of tumors may still retain copies of the wild-type *p53* gene. Recent evidence further suggests that, despite the production of functional wild-type p53 protein in human cancer cells, the amounts of wild-type p53 protein produced are so low that the protein, appears unable to execute its normal apoptotic function. In an effort to activate its apoptotic functions, it would therefore be of interest to determine the effect of fenbendazole and mebendazole on the trafficking of p53 protein between the cytoplasmic and nuclear compartment in these cells. Because phosphorylation of Ser 15 has been implicated as a mechanism underlying the increased stability of the p53 protein, the effect of these anthelmintics on the phosphorylation pattern of p53 is currently being investigated in our laboratory.

It is already known that DNA-damaging agents such as etoposide and Adriamycin induce production of the p53 protein in cell lines harboring the wild-type gene. These agents are

thought to enhance the level of p53 in cells by increasing its stability (23). This increase in stability has been borne out by immunofluorescence studies in fenbendazole-treated cells, which showed that there was a strong increase in p53 staining in the nucleus. Interestingly, our experiments also showed that the anthelmintic drugs we studied increased the half-life of p53 in H460 cells from 6 to 24 h.

The kinetics of p53 induction in H460 cells by these drugs and by MG132, a well-known proteasome inhibitor, are similar (data not shown). This strongly suggests that BZs inactivate a selective degradation pathway, thereby triggering the inductive effect on p53 in these cells. Therefore, it remains a possibility that the molecular target for BZs is not a kinase but a point in the p53-proteasome pathway of protein degradation where they can interfere with its progression.

This is the first report demonstrating that anthelmintics regulate the apoptotic function of wild-type p53 in human NSCLC cells. The fact that structurally related BZ analogues share this property of inducing apoptotic activities suggests that there is a unique and specific structural determinant of apoptosis embedded in their chemical structure and that this effect is mediated through wild-type p53. It has been suggested that the role of p53 in cell-cycle arrest may be distinct from its role in apoptosis and that each of these functions may be served by discrete domains in the molecule (24)(25). For example, a mutation in the p53 protein that inactivates its function in apoptosis may not necessarily affect its function in cell-cycle arrest. Interestingly, however, we observed in the present study that the induction of p53 protein in the drug-treated cells paralleled the induction of G1 cell-cycle arrest and apoptosis. It has been reported that Bax is a target gene for p53 (26). However, unlike p21, in the NSCLC wild-type p53 cell lines we studied here, the level of Bax protein, remained unchanged and was not upregulated by p53. Nevertheless, this result is consistent with the finding in some cell types that p53 does not appear to increase Bax levels (27).

The BZs are a group of structurally similar compounds that have been shown to possess antimitotic activity *in vitro* and *in vivo* (28) but also to induce a presumably p53-independent mechanism. In addressing this contradiction, we found no changes in the steady-state level of Bcl-2, RB, and Cdk-2 in H460 cells upon treatment with BZs for 24 h. However, it is possible that BZs regulate some of these proteins in a cell-type-dependent manner. In fact, the list of proteins that seem to play important roles in various apoptotic pathways is growing rapidly. However, whether BZs can regulate apoptotic genes other than those we have studied remains to be investigated.

In our study, fenbendazole and mebendazole induced p53 accumulation but failed to induce apoptosis in E6-expressing (HeLa and SiHa) cells. This inability may be attributed to the specific ubiquitination of E6-AP and the subsequent degradation of wild-type p53, actions that perhaps protect these cells from the effects of BZs. Alternatively, this inability may suggest the presence of an additional downstream step in the apoptotic pathway. To distinguish between these two possibilities, more human cancer cell lines carrying the wild-type p53 genes need to be evaluated.

Acknowledgments

We would like to thank Jude Richard for editorial review and Monica L. Contreras for assistance in preparing this manuscript.

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Figure Legends

Fig. 1. Induction of apoptosis. H460 cells were treated with 0.05 mg/ml mebendazole or fenbendazole and their morphological changes associated with apoptosis identified. A, widespread loss of viability was noted by gross examination of the cells at 24 h after treatment (upper panel). The cells were photographed using a phase-contrast light microscope. Chromatin condensation was noted under a fluorescence microscope at

24 h after treatment the condensation was shown by staining the nuclei with Hoechst 33342 fluorescent dye) (middle panel). DNA strand breaks were detected at 24 h after treatment using a direct immunoperoxidase method (lower panel). *B*, induction of apoptosis was assessed by TdT FACS analysis in the human lung cancer cell line H460. Cells were harvested after 24 h of exposure to fenbendazole and mebendazole (0.05 mg/ml) and the apoptotic cells quantitated after TdT staining as described in Experimental Procedures.

Fig. 2. Effect of drug treatment on the level of various proteins. Western blot analysis of various proteins in H460 cells before and after exposure to 0.05 µg/ml fenbendazole or mebendazole for 24 h. Whole-cell extracts were used for the analysis.

Fig. 3. Ability of fenbendazole and mebendazole to induce the expression of p53 protein and its target genes. *A*, H460 cells were treated with fenbendazole or mebendazole for 24 h (0.05 µg/ml), and the proteins from both control and treated cells were analyzed for p53 protein and p53 target gene expression. Increased p53 expression correlated with enhanced p21 and MDM2 protein levels. *B*, chemical structures of mebendazole and fenbendazole.

Fig. 4. Fenbendazole-and mebendazole-induced expression of p53 protein and mRNA. *A*, cell extracts were prepared from H460 cells exposed to fenbendazole (0.05 µg/ml) for various times (1 to 24 h). p53 protein was detected by immunoblot analysis. The position of the p53 protein is indicated by an arrow. *B*, northern blot analysis of p53 and p21 mRNA levels in H460 cells treated with 0.05 µg/ml fenbendazole or mebendazole after 24 h of treatment. Total RNA was extracted and used (20 µg per lane) for the

analysis.

Fig. 5. The effect of fenbendazole in inducing apoptosis. *A*, immunoblot analysis of p53 protein in the H460 human lung cancer cell line. The proteins were extracted from cells treated with different concentrations of fenbendazole and mebendazole for 24 h. ■ - represent FZ treated ; ▲ - represent control untreated cells. *B*, NSCLC cell lines of differing p53 status were treated with 0.05 µg/ml fenbendazole for 24 h and then stained with p53 monoclonal antibody. The nuclear accumulation of wild-type p53 was associated with nuclear fragmentation that resulted in apoptosis. (*A*, *C*, and *E*) Untreated control cells and fenbendazole Z-treated cells (*B*, *D*, and *F*); H460 (wild-type p53)--upper panel; H322 (mutated p53)--middle panel; H1299 (deleted p53)--lower panel

Fig. 6. Effect of fenbendazole and mebendazole on stability of the p53 protein. *A*, stability of the p53 protein in H460 cells upon fenbendazole and mebendazole treatment. H460 cells were treated with 0.05 µg/ml fenbendazole for 24 h, and then both untreated control and treated cells were washed with PBS and treated with 25 µg/ml cycloheximide. After that, cells were harvested at different times. The total cell protein was extracted and analyzed on a 10% SDS-polyacrylamide gel, followed by western blot analysis using p53 and actin monoclonal antibodies (actin served as an internal control). The experiment was repeated twice, producing similar results both times. *B*, p53 protein stability in cells. Data from the pulse-chase experiments were quantitated with a PhosphorImager using ImageQuant™ software. Circles and triangles represent data from fenbendazole-treated and control samples, respectively.

Fig. 7. Effect of fenbendazole and mebendazole the induction of cell growth and nuclear accumulation of wild-type p53 protein. A, The NSCLC cell lines H460 (wild-type p53), H322 (mutant p53), and H1299 (deleted p53) were exposed to fenbendazole (filled bar) or mebendazole (hatched bar) at concentrations of 0.05 $\mu\text{g/ml}$. The cells were plated onto six-well plates and their viability determined by trypan blue extrusion. Cells were then counted on a hemocytometer. The values shown are the means \pm standard deviation of triplicate samples. Duplicate experiments gave similar results. Using equal amounts of protein extracts taken from each cell line before and after treatment, an immunoblot analysis of nuclear p53 was also performed to determine whether these drugs induced the production of p53. The upper band corresponds to p53, and the lower band corresponds to actin protein, which was used as an internal loading control.

Fig. 8 Synergistic effect of Ad5p53 and Fenbendazole. Approximately 10^4 cells were seeded on tissue culture plates 24 h before drug treatment or adenoviral infection. A) Cell growth was measured in untreated controls, cells treated with 0.05ng/ml FZ and cells treated with the combination of Ad5p53 (1 MOI) and FZ . B) H322 cells were also treated with an empty adenoviral vector, dl312, in addition to Adp53 to show dl312 had no effect alone or in combination with FZ. C) A549 lung cancer cells were examined for apoptotic cell death 48 h after FZ and Adp53 treatment alone or in combination. Percent apoptotic cells were measured by TdT-FACS analysis.

Fig. 9 Effect of E6 protein on the cytotoxicity of fenbendazole. Production of p53, MDM2, and p21 proteins after fenbendazole treatment was examined in HeLa and SiHa cells and compared with the production of these proteins in H460 cells. The positions of the p53, MDM2, and p21 protein bands are indicated.

Table I. Sensitivity of cancer cell lines to bendimidazoles

Phenotype and cell lines	IC ₅₀	
	Fenbendazole (nM)	Mebendazole (nM)
p53 positive		
H460	152	106
A549	123	130
HeLa (p53 inactivated)	853	400
p53 mutated		
H322	816	871
H596	643	601
p53 deleted		
H358	654	893

^aConcentrations of drugs (in nanomolars) required to inhibit growth by 50% after 1 day of exposure.

Table II. Effect of fenbendazole and mebendazole on p53 induction and apoptosis in human tumor cell lines of differing p53 status^a

Cell Line	Tumor origin	p53 status	% Cell Viability		Nuclear p53	p53 Induction
			fenbendazole ^b	mebendazole ^b		
MCF-7	Breast	Wild-type (29)C	43.88 ± 7.3	24.26 ± 8.4	No	Yes
H460	Lung	Wild-type (30)C	39.16 ± 6.4	25.23 ± 7.6	Yes	Yes
H549	Lung	Wild-type (30)C	46.43 ± 13.3	39.19 ± 10.7	Yes	Yes
H322	Lung	R248H (30)	65.92 ± 4.9	68.50 ± 3.7	Yes	No
H596	Lung	R245C (30)	86.71 ± 11.5	86.19 ± 8.0	Yes	No
H226Br	Lung	R254 (31)	80.06 ± 11.1	64.24 ± 0.2	Yes	No
H1299	Lung	Deleted (30)	89.17 ± 1.1	89.18 ± 4.3	No	No
H358	Lung	Deleted (30)	88.35 ± 17.8	73.45 ± 1.8	No	No
Saos-2	Osteosarcoma	Deleted (32)	76.48 ± 5.9	65.36 ± 10.1	No	No
Hep 3B	Liver	Deleted (33)	79.49 ± 12.5	83.24 ± 1.5	No	No
SW480	Colon	R273H (21)	70.59 ± 7.6	63.63 ± 13.3	Yes	No
MDA 231	Breast	R280K (34)	65.26 ± 5.0	66.24 ± 15.8	Yes	No
SK-OV-433	Ovarian	Wild-type (35)C	52.52 ± 13.2	28.15 ± 6.1	Yes	Yes
HeLa	Cervical	Wild-type but inactivated by E6 (34)C	93.63 ± 7.4	92.42 ± 6.27	Yes	No

SiHa	Cervical	Wild-type but inactivated by E6 (36) ^c	94.52 ± 7.4	91.32 ± 8.6	Yes	No
RD	Rhabdomyosarcom a	R248W (37)	78.73 ± 12.0	72.24 ± 2.8	Yes	No
HT1080	Osteosarcoma	Wild-type (38) ^c	54.52 ± 12.0	44.49 ± 7.7	ND ^d	ND ^d

^aThe viability of the cells was measured by trypan blue extrusion cell count assay. The 100% value was derived from measurements obtained from untreated cells. Experiments were done in triplicate. p53 protein was examined by immunoblot analysis of the nuclear extracts isolated from each cell line before and after treatment with 0.05 mg/ml fenbendazole and mebendazole for 24 h.

^bConcentration of fenbendazole and mebendazole = 0.05 mg/ml.

^cThis cell line carries a wild-type allele.

^dND, not done.

EXHIBIT 2

Page No. 178

Cell) HCT116 (P31+) HCT116 (P37-) A549

Design) (day 0) cell preparation 1000/well/well media (A549), 3000/well (HCT)

(day 1) Add 50ul of media containing MZ or DMSO

(day 3) XTT assay

Results)

HCT116(-/-) treated with MZ or DMSO; 48 hours; XTT; 1107-1110000

DMSO(%)	0	0.0005	0.001	0.0025	0.005	0.01	0.025	0.05	0.1	0.25	0.5	
1	0.817	1.124	1.128	1.169	1.26	1.223	1.192	1.088	1.289	1.223	1.266	0.7
2	1.14	1.207	1.2	1.148	1.224	1.214	1.222	1.283	1.287	1.293	1.249	1.0
3	1.129	1.209	1.189	1.2	1.263	1.241	1.152	1.213	1.214	1.231	1.346	1.1
4	1.08	1.203	1.123	1.175	1.329	1.204	1.115	1.142	1.214	1.243	1.294	0.9
5	0.542	1.157	1.13	1.16	1.205	1.292	1.232	1.232	1.254	1.215	1.182	0.6
Average	0.9416	1.18	1.154	1.1704	1.256	1.2348	1.1826	1.1916	1.2516	1.241	1.2674	0.90
SD	0.2319	0.034	0.0333	0.0174	0.042	0.0311	0.0438	0.0688	0.0331	0.0276	0.0539	0.198
MZ(ug/ml)	0	0.005	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	
1	0.835	0.998	0.971	1.01	1.159	1.043	0.982	0.807	0.603	0.325	0.223	0.16
2	1.111	1.129	1.101	1.149	1.095	1.185	1.147	0.844	0.628	0.357	0.268	0.18
3	1.082	1.142	1.077	1.144	1.174	1.148	1.099	0.942	0.544	0.369	0.259	0.17
4	1.028	1.143	1.179	1.218	1.222	1.217	1.172	0.857	0.586	0.346	0.239	0.16
5	0.645	1.039	1.107	1.235	1.267	1.308	1.198	0.957	0.644	0.348	0.221	0.11
Average	0.9402	1.0902	1.087	1.1512	1.183	1.1802	1.1196	0.8814	0.601	0.349	0.242	0.1626
SD	0.1762	0.0602	0.0673	0.0793	0.058	0.0867	0.0762	0.0582	0.0348	0.0145	0.0188	0.0273

Average	0	0.005	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
DMSO	0.9416	1.18	1.154	1.1704	1.256	1.2348	1.1826	1.1916	1.2516	1.241	1.2674	0.9068
MZ	0.9402	1.0902	1.087	1.1512	1.183	1.1802	1.1196	0.8814	0.601	0.349	0.242	0.1626
Ratio	0	0.005	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
DMSO	1	1.2532	1.2256	1.243	1.334	1.3114	1.2559	1.2655	1.3292	1.318	1.346	0.963
MZ	1	1.1595	1.1561	1.2244	1.259	1.2553	1.1908	0.9375	0.6392	0.3712	0.2574	0.1729

To Page No. _____

Used & Understood by me,

Yoshi

Date

6/5/01

Invented by

Hiroyuki Sasaki

Date

11/10/00

Recorded by

Hiroyuki Sasaki

From Page No. _____

Sample : H460, HCT116 P53 +/+ , HCT116 P53 -/- (Whole cell lysate and Cytosolic/Mitochondrial)

Treatment : MZ 0.05 μ g/ml 24 hours

Lysis : 1x SDS, 2ME

Volume of apply : 50 μ g for Metallothionein, 10 μ g for cytochrome C.

PAGE : 12.5 %

First antibody Metallothionein x 200
Cytochrome C x 500) 1 hour at r.t.

Second antibody Anti-mouse HRP x 2000 1 hour at r.t.

Exposure 15 sec & 5 min

Re-probe P53 & β -actin (P53 x 2000, β -actin x 5000)

To Page No. _____

Witnessed & Understood by me,

yoshi

Date

6/5/01

Invented by

Toshio Sasaki

Date

11/6/01

Recorded by A. D. D. D.

Project No. 5Book No. 1TITLE XTTFrom Page No.

To make Dose response curve after treatment MZ.

Cell: H460, A549, HCT116/-

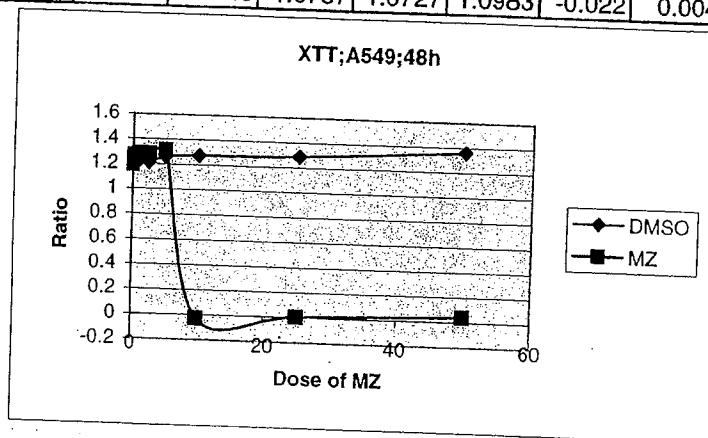
Treatment: MZ (0 - 50 μ M) 48 hours exposure

Method: XTT (2h)

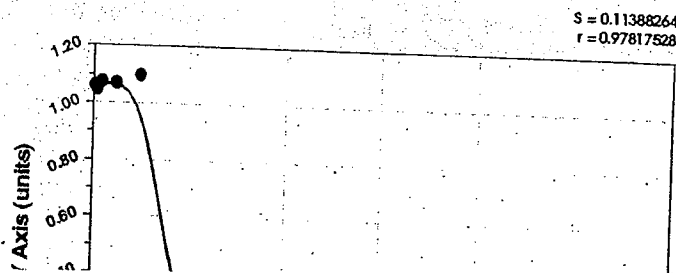
A549 treated with MZ or DMSO; 48 hours; XTT; 1201-120500

DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	1.264	1.18	1.154	1.22	1.206	1.204	1.196	1.262	1.224	1.277	1.361
2	1.239	1.193	1.153	1.202	1.203	1.205	1.177	1.221	1.249	1.257	1.343
3	1.299	1.19	1.17	1.241	1.245	1.24	1.196	1.243	1.282	1.269	1.332
4	1.321	1.248	1.298	1.364	1.258	1.256	1.275	1.31	1.334	1.348	1.392
Average	1.2808	1.2028	1.1938	1.2568	1.228	1.2263	1.211	1.259	1.2723	1.2878	1.357
SD	0.0315	0.0266	0.0606	0.0634	0.024	0.0225	0.0378	0.0328	0.0412	0.0355	0.0227
MZ(μ M)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	1.159	1.231	1.302	1.283	1.316	1.341	1.389	1.394	-0.006	0.016	0.073
2	1.199	1.289	1.275	1.311	1.265	1.339	1.285	1.341	-0.022	0.011	0.039
3	1.195	1.202	1.205	1.231	1.204	1.21	1.24	1.26	-0.039	-0.003	0.035
4	1.22	1.205	1.193	1.238	1.209	1.235	1.206	1.247	-0.04	-0.005	0.02
Average	1.1933	1.2318	1.2438	1.2658	1.249	1.2813	1.28	1.3105	-0.027	0.0048	0.0418
SD	0.0219	0.0349	0.046	0.0329	0.046	0.0594	0.0689	0.0602	0.014	0.009	0.0194

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1.2808	1.2028	1.1938	1.2568	1.228	1.2263	1.211	1.259	1.2723	1.2878	1.357
MZ	1.1933	1.2318	1.2438	1.2658	1.249	1.2813	1.28	1.3105	-0.027	0.0048	0.0418
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	0.9391	0.9321	0.9813	0.959	0.9574	0.9455	0.983	0.9934	1.0055	1.0595
MZ	1	1.0323	1.0423	1.0608	1.046	1.0737	1.0727	1.0983	-0.022	0.004	0.035



0	1
0.05	1.0323
0.1	1.0423
0.25	1.0608
0.5	1.0463
1	1.0737
2.5	1.0727
5	1.0983
10	-0.022



To make Dose response curve after treatment MZ

Cell: H460, A549, HCT116/-

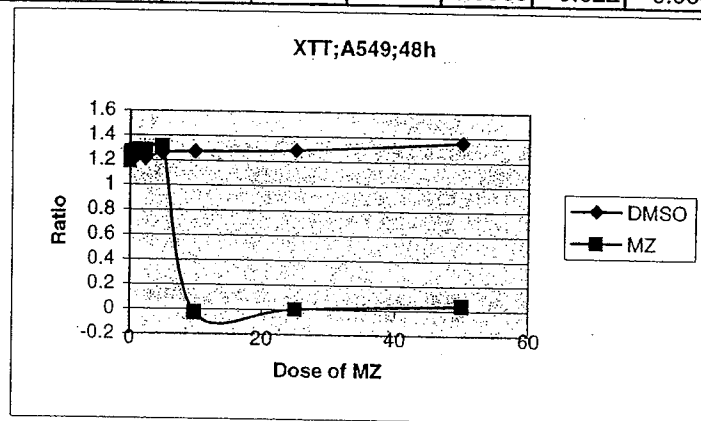
Treatment: MZ (0 - 50 μ M) 48 hours exposure

Method: XTT (2h)

A549 treated with MZ or DMSO; 48 hours; XTT; 1201-120500

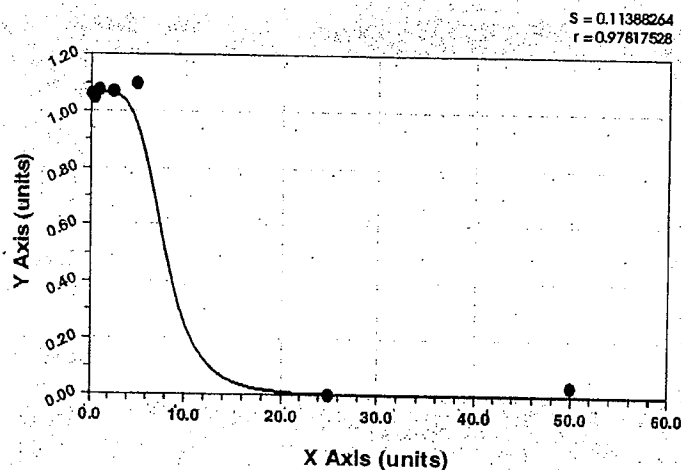
DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	1.264	1.18	1.154	1.22	1.206	1.204	1.196	1.262	1.224	1.277	1.361
2	1.239	1.193	1.153	1.202	1.203	1.205	1.177	1.221	1.249	1.257	1.343
3	1.299	1.19	1.17	1.241	1.245	1.24	1.196	1.243	1.282	1.269	1.332
4	1.321	1.248	1.298	1.364	1.258	1.256	1.275	1.31	1.334	1.348	1.392
Average	1.2808	1.2028	1.1938	1.2568	1.228	1.2263	1.211	1.259	1.2723	1.2878	1.357
SD	0.0315	0.0266	0.0606	0.0634	0.024	0.0225	0.0378	0.0328	0.0412	0.0355	0.0227
MZ(μ M)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	1.159	1.231	1.302	1.283	1.316	1.341	1.389	1.394	-0.006	0.016	0.073
2	1.199	1.289	1.275	1.311	1.265	1.339	1.285	1.341	-0.022	0.011	0.039
3	1.195	1.202	1.205	1.231	1.204	1.21	1.24	1.26	-0.039	-0.003	0.035
4	1.22	1.205	1.193	1.238	1.209	1.235	1.206	1.247	-0.04	-0.005	0.02
Average	1.1933	1.2318	1.2438	1.2658	1.249	1.2813	1.28	1.3105	-0.027	0.0048	0.0418
SD	0.0219	0.0349	0.046	0.0329	0.046	0.0594	0.0689	0.0602	0.014	0.009	0.0194

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1.2808	1.2028	1.1938	1.2568	1.228	1.2263	1.211	1.259	1.2723	1.2878	1.357
MZ	1.1933	1.2318	1.2438	1.2658	1.249	1.2813	1.28	1.3105	-0.027	0.0048	0.0418
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	0.9391	0.9321	0.9813	0.959	0.9574	0.9455	0.983	0.9934	1.0055	1.0595
MZ	1	1.0323	1.0423	1.0608	1.046	1.0737	1.0727	1.0983	-0.022	0.004	0.035



	0	1
	0.05	1.0323
	0.1	1.0423
	0.25	1.0608
	0.5	1.0463
	1	1.0737
	2.5	1.0727
	5	1.0983
	10	-0.022
	25	0.004
	50	0.035

IC20	6.16
IC50	7.97
IC80	10.6



To make Dose response curve after treatment MZ.

Cell: H460, A549, HCT116/-

Treatment: MZ (0 - 50 μ M) 48 hours exposure

Method: XTT (6h)

A549 treated with MZ or DMSO; 48 hours; XTT; 1201-120500

DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	1.264	1.18	1.154	1.22	1.206	1.204	1.196	1.262	1.224	1.277	1.361
2	1.239	1.193	1.153	1.202	1.203	1.205	1.177	1.221	1.249	1.257	1.343
3	1.299	1.19	1.17	1.241	1.245	1.24	1.196	1.243	1.282	1.269	1.332
4	1.321	1.248	1.298	1.364	1.258	1.256	1.275	1.31	1.334	1.348	1.392
Average	1.2808	1.2028	1.1938	1.2568	1.228	1.2263	1.211	1.259	1.2723	1.2878	1.357
SD	0.0315	0.0266	0.0606	0.0634	0.024	0.0225	0.0378	0.0328	0.0412	0.0355	0.0227
MZ(μ M)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	1.159	1.231	1.302	1.283	1.316	1.341	1.389	1.394	-0.006	0.016	0.073
2	1.199	1.289	1.275	1.311	1.265	1.339	1.285	1.341	-0.022	0.011	0.039
3	1.195	1.202	1.205	1.231	1.204	1.21	1.24	1.26	-0.039	-0.003	0.035
4	1.22	1.205	1.193	1.238	1.209	1.235	1.206	1.247	-0.04	-0.005	0.02
Average	1.1933	1.2318	1.2438	1.2658	1.249	1.2813	1.28	1.3105	-0.027	0.0048	0.0418
SD	0.0219	0.0349	0.046	0.0329	0.046	0.0594	0.0689	0.0602	0.014	0.009	0.0194

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1.2808	1.2028	1.1938	1.2568	1.228	1.2263	1.211	1.259	1.2723	1.2878	1.357
MZ	1.1933	1.2318	1.2438	1.2658	1.249	1.2813	1.28	1.3105	-0.027	0.0048	0.0418
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	0.9391	0.9321	0.9813	0.959	0.9574	0.9455	0.983	0.9934	1.0055	1.0595
MZ	1	1.0323	1.0423	1.0608	1.046	1.0737	1.0727	1.0983	-0.022	0.004	0.035

To Page No.

Read & Understood by me,

yoshi

Date

6/5/01

Invented by

J.S

Date

12/5/00

Recorded by

J.S

From Page No. — To make Dose response curve and evaluate synergistic effect of Radiation

Cell: H460, A549

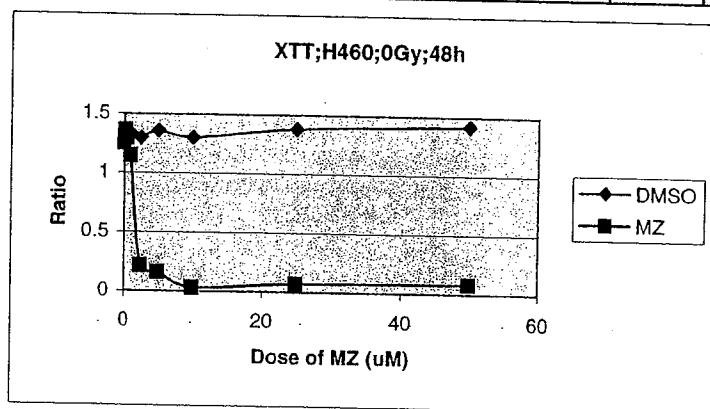
Treatment: Radiation 0G, 5Gy, 10Gy, MZ 0-50uM, 48 hours

Method: XTT (2h)

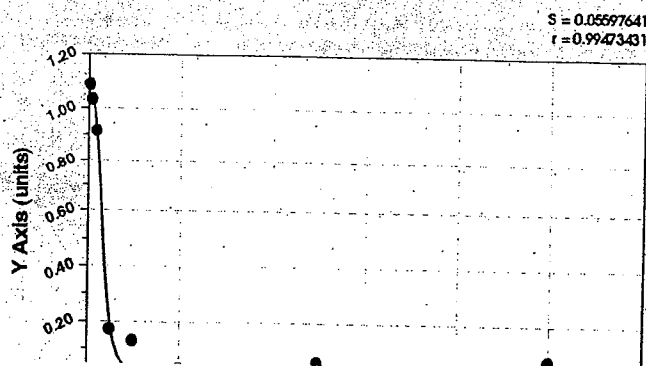
H460 treated with MZ and Radiation; Radiation: 0Gy 48 hours; XTT; 1201-120500

DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	1.288	1.284	1.19	1.257	1.255	1.347	1.336	1.359	1.309	1.419	1.431
2	1.228	1.265	1.244	1.251	1.254	1.274	1.25	1.312	1.271	1.344	1.381
3	1.284	1.288	1.238	1.288	1.346	1.344	1.359	1.415	1.345	1.343	1.405
4	1.273	1.283	1.371	1.341	1.293	1.352	1.241	1.349	1.279	1.406	1.455
Average	1.2683	1.28	1.2608	1.2843	1.287	1.3293	1.2965	1.3588	1.301	1.378	1.418
SD	0.0239	0.0089	0.067	0.0356	0.038	0.032	0.0517	0.0369	0.0291	0.0348	0.0277
MZ(uM)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	1.207	1.243	1.277	1.342	1.34	1.049	0.172	0.165	0.036	0.072	0.106
2	1.277	1.312	1.312	1.422	1.332	1.084	0.208	0.137	0.033	0.073	0.081
3	1.256	1.302	1.351	1.366	1.258	1.104	0.247	0.175	0.03	0.064	0.077
4	1.275	1.331	1.289	1.328	1.263	1.353	0.236	0.172	0.037	0.069	0.068
Average	1.2538	1.297	1.3073	1.3645	1.298	1.1475	0.2158	0.1623	0.034	0.0695	0.083
SD	0.0282	0.0329	0.0282	0.0359	0.038	0.1203	0.029	0.015	0.0027	0.0035	0.0141

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1.2683	1.28	1.2608	1.2843	1.287	1.3293	1.2965	1.3588	1.301	1.378	1.418
MZ	1.2538	1.297	1.3073	1.3645	1.298	1.1475	0.2158	0.1623	0.034	0.0695	0.083
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	1.0093	0.9941	1.0126	1.015	1.0481	1.0223	1.0714	1.0258	1.0865	1.1181
MZ	1	1.0345	1.0427	1.0883	1.036	0.9153	0.1721	0.1294	0.0271	0.0554	0.0662



0	1
0.05	1.0345
0.1	1.0427
0.25	1.0883
0.5	1.0355
1	0.9153
2.5	0.1721
5	0.1294
10	0.0271
25	0.0554
50	0.0662



Radiation

Cell: H460, A549

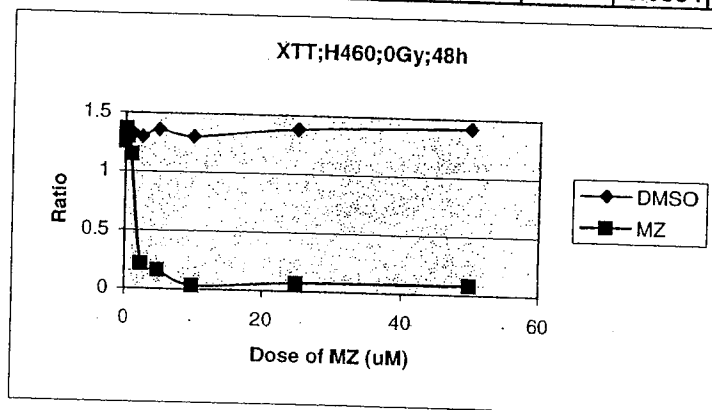
Treatment: Radiation 0G, 5G, 10G, MZ 0-50uM, 48 hours

Method: XTT (2h)

H460 treated with MZ and Radiation; Radiation: 0Gy 48 hours; XTT: 1201-120500

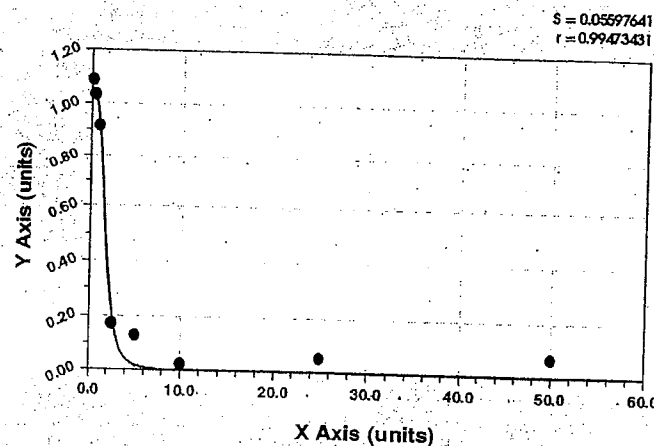
DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	1.288	1.284	1.19	1.257	1.255	1.347	1.336	1.359	1.309	1.419	1.431
2	1.228	1.265	1.244	1.251	1.254	1.274	1.25	1.312	1.271	1.344	1.381
3	1.284	1.288	1.238	1.288	1.346	1.344	1.359	1.415	1.345	1.343	1.405
4	1.273	1.283	1.371	1.341	1.293	1.352	1.241	1.349	1.279	1.406	1.455
Average	1.2683	1.28	1.2608	1.2843	1.287	1.3293	1.2965	1.3588	1.301	1.378	1.418
SD	0.0239	0.0089	0.067	0.0356	0.038	0.032	0.0517	0.0369	0.0291	0.0348	0.0277
MZ(uM)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	1.207	1.243	1.277	1.342	1.34	1.049	0.172	0.165	0.036	0.072	0.106
2	1.277	1.312	1.312	1.422	1.332	1.084	0.208	0.137	0.033	0.073	0.081
3	1.256	1.302	1.351	1.366	1.258	1.104	0.247	0.175	0.03	0.064	0.077
4	1.275	1.331	1.289	1.328	1.263	1.353	0.236	0.172	0.037	0.069	0.068
Average	1.2538	1.297	1.3073	1.3645	1.298	1.1475	0.2158	0.1623	0.034	0.0695	0.083
SD	0.0282	0.0329	0.0282	0.0359	0.038	0.1203	0.029	0.015	0.0027	0.0035	0.0141

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1.2683	1.28	1.2608	1.2843	1.287	1.3293	1.2965	1.3588	1.301	1.378	1.418
MZ	1.2538	1.297	1.3073	1.3645	1.298	1.1475	0.2158	0.1623	0.034	0.0695	0.083
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	1.0093	0.9941	1.0126	1.015	1.0481	1.0223	1.0714	1.0258	1.0865	1.1181
MZ	1	1.0345	1.0427	1.0883	1.036	0.9153	0.1721	0.1294	0.0271	0.0554	0.0662



0	1
0.05	1.0345
0.1	1.0427
0.25	1.0883
0.5	1.0355
1	0.9153
2.5	0.1721
5	0.1294
10	0.0271
25	0.0554
50	0.0662

IC20	1.2
IC50	1.7
IC80	2.5



Form Page No.

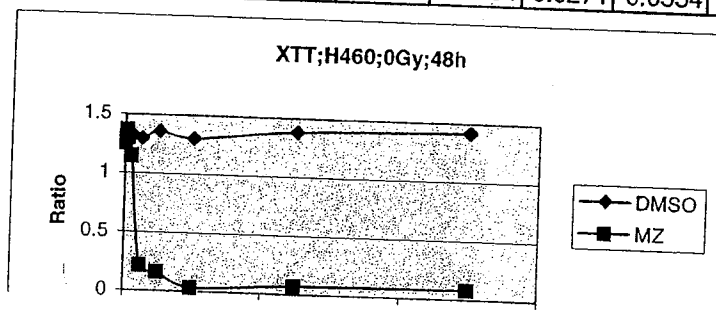
To make Dose response curve and evaluate synergistic effect of MZ and

Radiation
Cell: H460, A549Treatment: Radiation 0G, 5Gy, 10Gy, MZ 0-50 μ M, 48 hours
Method: XTT (2h)

H460 treated with MZ and Radiation; Radiation: 0Gy 48 hours; XTT; 1201-120500

DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	1.288	1.284	1.19	1.257	1.255	1.347	1.336	1.359	1.309	1.419	1.431
2	1.228	1.265	1.244	1.251	1.254	1.274	1.25	1.312	1.271	1.344	1.381
3	1.284	1.288	1.238	1.288	1.346	1.344	1.359	1.415	1.345	1.343	1.405
4	1.273	1.283	1.371	1.341	1.293	1.352	1.241	1.349	1.279	1.406	1.455
Average	1.2683	1.28	1.2608	1.2843	1.287	1.3293	1.2965	1.3588	1.301	1.378	1.418
SD	0.0239	0.0089	0.067	0.0356	0.038	0.032	0.0517	0.0369	0.0291	0.0348	0.0277
MZ(μ M)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	1.207	1.243	1.277	1.342	1.34	1.049	0.172	0.165	0.036	0.072	0.106
2	1.277	1.312	1.312	1.422	1.332	1.084	0.208	0.137	0.033	0.073	0.081
3	1.256	1.302	1.351	1.366	1.258	1.104	0.247	0.175	0.03	0.064	0.077
4	1.275	1.331	1.289	1.328	1.263	1.353	0.236	0.172	0.037	0.069	0.068
Average	1.2538	1.297	1.3073	1.3645	1.298	1.1475	0.2158	0.1623	0.034	0.0695	0.083
SD	0.0282	0.0329	0.0282	0.0359	0.038	0.1203	0.029	0.015	0.0027	0.0035	0.0141

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1.2683	1.28	1.2608	1.2843	1.287	1.3293	1.2965	1.3588	1.301	1.378	1.418
MZ	1.2538	1.297	1.3073	1.3645	1.298	1.1475	0.2158	0.1623	0.034	0.0695	0.083
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	1.0093	0.9941	1.0126	1.015	1.0481	1.0223	1.0714	1.0258	1.0865	1.1181
MZ	1	1.0345	1.0427	1.0883	1.036	0.9153	0.1721	0.1294	0.0271	0.0554	0.0662



Inspected & Understood by me,

yoshi

Date

6/5/01

Invented by

J-S

Recorded by

J-S

Date

12/5/00

To Page No.

From Page No.

Dose response curve after MZ treatment and CD Treatment

All - H1299

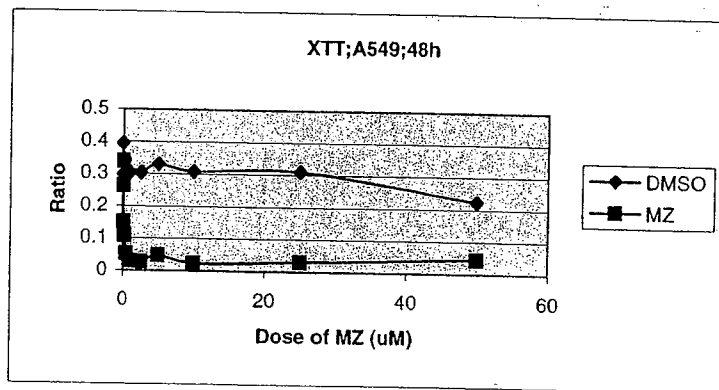
Treatment: MZ (0-50 μ M) 48 hours exposure CD (0-500 μ M)

Method: XTT

H1299 treated with MZ or DMSO; 48 hours; XTT; 1205-120800

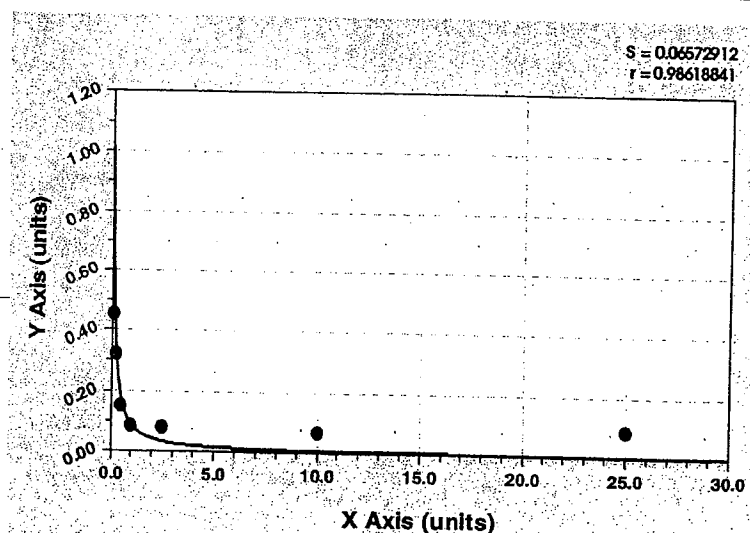
DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	0.321	0.329	0.328	0.373	0.341	0.326	0.311	0.338	0.29	0.32	0.233
2	0.38	0.307	0.292	0.329	0.309	0.304	0.294	0.278	0.32	0.336	0.216
3	0.446	0.338	0.255	0.323	0.259	0.28	0.302	0.322	0.284	0.269	0.216
4	0.43	0.369	0.32	0.34	0.253	0.303	0.306	0.382	0.336	0.315	0.228
Average	0.3943	0.3358	0.2988	0.3413	0.291	0.3033	0.3033	0.33	0.3075	0.31	0.2233
SD	0.0488	0.0223	0.0286	0.0193	0.036	0.0163	0.0062	0.0372	0.0214	0.0249	0.0075
MZ(μ M)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	0.325	0.26	0.182	0.118	0.068	0.044	0.035	0.04	0.045	0.039	0.092
2	0.353	0.274	0.138	0.099	0.052	0.032	0.042	0.043	0.024	0.037	0.039
3	0.346	0.263	0.143	0.092	0.062	0.015	0.023	0.041	0.013	0.029	0.034
4	0.329	0.258	0.146	0.127	0.025	0.025	0.01	0.037	0.011	0.014	0.015
Average	0.3383	0.2638	0.1523	0.109	0.052	0.029	0.0275	0.0403	0.0233	0.0298	0.045
SD	0.0116	0.0062	0.0174	0.0141	0.016	0.0106	0.0122	0.0022	0.0135	0.0098	0.0286

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	0.3943	0.3358	0.2988	0.3413	0.291	0.3033	0.3033	0.33	0.3075	0.31	0.2233
MZ	0.3383	0.2638	0.1523	0.109	0.052	0.029	0.0275	0.0403	0.0233	0.0298	0.045
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	0.8516	0.7578	0.8656	0.737	0.7692	0.7692	0.837	0.78	0.7863	0.5663
MZ	1	0.7797	0.4501	0.3222	0.153	0.0857	0.0813	0.1426	0.0687	0.088	0.133



0	1
0.05	0.7797
0.1	0.4501
0.25	0.3222
0.5	0.153
1	0.0857
2.5	0.0813
10	0.0687
25	0.088

IC20	0.03
IC50	0.11
IC80	0.4



No.

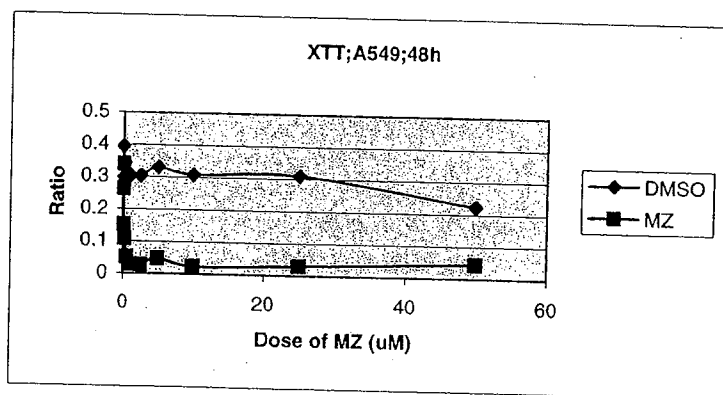
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Dose response curve after MZ treatment and CD Treatment
 All: H1299
 Treatment: MZ (0 - 50 μ M) 48 hours exposure CD (0 - 500 μ M)
 Method: XTT

H1299 treated with MZ or DMSO; 48 hours; XTT; 1205-120800

DMSO(%)	0	0.0015	0.003	0.0075	0.015	0.03	0.075	0.15	0.3	0.75	1.5
1	0.321	0.329	0.328	0.373	0.341	0.326	0.311	0.338	0.29	0.32	0.233
2	0.38	0.307	0.292	0.329	0.309	0.304	0.294	0.278	0.32	0.336	0.216
3	0.446	0.338	0.255	0.323	0.259	0.28	0.302	0.322	0.284	0.269	0.216
4	0.43	0.369	0.32	0.34	0.253	0.303	0.306	0.382	0.336	0.315	0.228
Average	0.3943	0.3358	0.2988	0.3413	0.291	0.3033	0.3033	0.33	0.3075	0.31	0.2233
SD	0.0488	0.0223	0.0286	0.0193	0.036	0.0163	0.0062	0.0372	0.0214	0.0249	0.0075
MZ(μ M)	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
1	0.325	0.26	0.182	0.118	0.068	0.044	0.035	0.04	0.045	0.039	0.092
2	0.353	0.274	0.138	0.099	0.052	0.032	0.042	0.043	0.024	0.037	0.039
3	0.346	0.263	0.143	0.092	0.062	0.015	0.023	0.041	0.013	0.029	0.034
4	0.329	0.258	0.146	0.127	0.025	0.025	0.01	0.037	0.011	0.014	0.015
Average	0.3383	0.2638	0.1523	0.109	0.052	0.029	0.0275	0.0403	0.0233	0.0298	0.045
SD	0.0116	0.0062	0.0174	0.0141	0.016	0.0106	0.0122	0.0022	0.0135	0.0098	0.0286

Average	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	0.3943	0.3358	0.2988	0.3413	0.291	0.3033	0.3033	0.33	0.3075	0.31	0.2233
MZ	0.3383	0.2638	0.1523	0.109	0.052	0.029	0.0275	0.0483	0.0233	0.0298	0.045
Ratio	0	0.05	0.1	0.25	0.5	1	2.5	5	10	25	50
DMSO	1	0.8516	0.7578	0.8656	0.737	0.7692	0.7692	0.837	0.78	0.7863	0.5663
MZ	1	0.7797	0.4501	0.3222	0.153	0.0857	0.0813	0.1426	0.0687	0.088	0.133



0 1

nessed & Understood by me,

yoshi

Date

6/5/01

Invented by

J-S

Recorded by

J-S

Date

6/8/00

To Page No. _____

From Page No. _____

Cell: A549

Treatment: MZ 10 μ M, time course (0h, 12h, 24h, 36h)

Lysate: SDS + Urea

Volume: 50 μ g

PAGE: 10 %

1st Ab: caspase 3 (x500)

2nd Ab: Anti-rabbit HRP (x5000) } 1h, rt.

Exposure: 5 sec and 30 sec

Re-probe: P53 & β -actin

Witnessed & Understood by me,

yoshi

Date

6/5/01

Invented by J.S

Recorded by J.S

Date

12/20/00

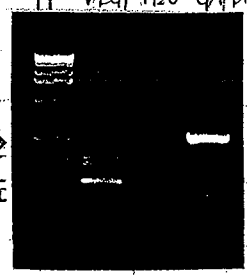
To Page No. _____

FILE RT-PCR

Page No. VEGF expression after MZ treatment
Cell: H1299, A549, H358
Treatment: MZ; 24 hours

H1299
M VEGF H₂O GAPDH

517
506
396
348
220
201



← Type I VEGF
(293 bp)

VEGF

BECKMAN DU-800

Date: 12/20/00
Time: 05:33

Nucleic Acid
ReadSamples Method SaveClear Print Quit

Results file: A:\WORK_RES Method name: A:\RNA100

Assay type: General Ratio and Concentration Units: ug/ml
Formula setup: VIEW Background Correction: [No]
Sampling device: None Concentration: [Yes]
Read average time: 0.50 sec Peak Pick: [No]

Sample ID	abs 260.0 nm	abs 280.0 nm	260.0 nm 280.0 nm	Protein ug/ml	Nucleic Acid ug/ml
1	N	0.3232	0.2021	1.5993	0.6253
2	0.05	0.3368	0.2130	1.5809	0.6325
3	0.1	0.3761	0.2364	1.5909	0.6286
4	0.5	0.2643	0.1645	1.6067	0.6224
5	N	0.4051	0.2456	1.6493	0.6063
6	0.05	0.3709	0.2234	1.6603	0.6023
7	0.1	0.4368	0.2643	1.6527	0.6051
8	0.5	0.3481	0.2126	1.6371	0.6108
				0.0000	1292.8983
				0.0000	1347.1895
				0.0000	1504.3396
				0.0000	1057.1166
				0.0000	1620.2367
				0.0000	1483.5201
				0.0000	1747.2542
				0.0000	1392.2246

A549

Sample ID	abs 260.0 nm	abs 280.0 nm	260.0 nm 280.0 nm	Protein ug/ml	Nucleic Acid ug/ml
1	N	0.2022	0.1297	1.5591	0.6414
2	0.5	0.2110	0.1376	1.5328	0.6524
3	1	0.1591	0.1033	1.5401	0.6493
4	5um	0.1939	0.1280	1.5148	0.6602
				0.0000	808.8824
				0.0000	843.8287
				0.0000	636.2218
				0.0000	775.4238

Witnessed & Understood by me,

yoshi

Date 6/5/01 Invented by J-S
Recorded by J-S

To Page No. 2

Date 12/22

age No. To make Dose response curve of MZ treatment,
 ① Cell count
 ② XTT assay was done.

ll: H460, H1299,

treatment: MZ (0 - 5 μ M: cell count, 0 - 10 μ M: XTT)
 24, 48, 72 hours exposure

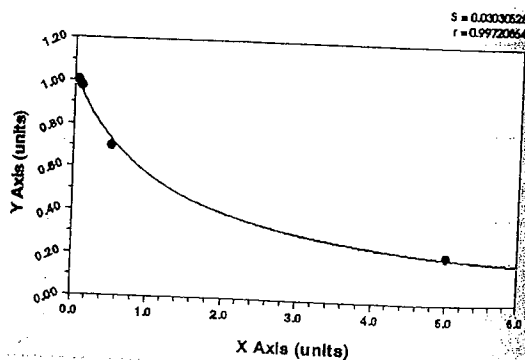
Cells were treated with MZ; 24 hours; Cell count; 0107-011101

H1299	0	0.01	0.05	0.1	0.5	5
1	3.38	3	2.88	3	2	0.75
2	2.88	3.38	2.75	3.38	2.5	0.63
3	2.88	2.75	3.5	2.5	1.88	0.63
Average	3.0467	3.0433	3.0433	2.96	2.127	0.67
SD	0.2357	0.259	0.3272	0.3604	0.268	0.0566
H460	0	0.01	0.05	0.1	0.5	5
1	5.5	6.38	6.63	6.13	2.13	0.38
2	6.63	6.25	6.25	4.25	1.75	0.5
3	7.88	7.13	6.25	5	1.25	0.5
Average	6.67	6.5867	6.3767	5.1267	1.71	0.46
SD	0.972	0.3878	0.1791	0.7727	0.36	0.0566

Average	0	0.01	0.05	0.1	0.5	5
h1299	3.0467	3.0433	3.0433	2.96	2.127	0.67
h460	6.67	6.5867	6.3767	5.1267	1.71	0.46
Ratio	0	0.25	0.5	1	2.5	10
h1299	1	0.9989	0.9989	0.9716	0.698	0.2199
h460	1	0.9875	0.956	0.7686	0.256	0.069

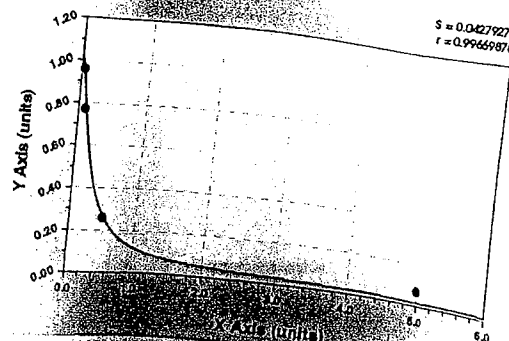
Ratio	h1299
0	1
0.01	0.9989
0.05	0.9989
0.1	0.9716
0.5	0.698
5	0.2199

IC20	0.33
IC50	1.29
IC80	5.2



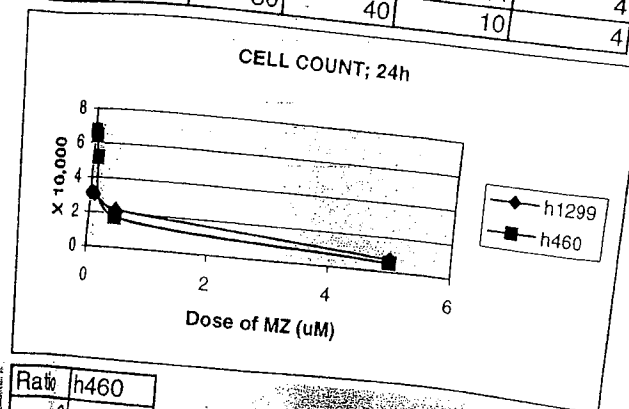
Ratio	h460
0	1
0.01	0.9875
0.05	0.956
0.1	0.7686
0.5	0.2564
5	0.069

IC20	0.1
IC50	0.24
IC80	0.63



27	24	23	24	16	6
23	27	22	27	20	5
23	22	28	20	15	5

44	51	53	49	17	3
53	50	50	34	14	4
63	57	50	40	10	4



& Understood by me,

Yoshi

Date

6/5/01

Invented by

JS

Recorded by

JS

Date

1/11/01

age No. VEGF - RT-PCR after MZ treatmentCell: H1299, H460Treatment: MZ Dose (0, 0.05, 0.1, 0.5, 1 μ M) Time (0, 1, 2, 6, 12, 24 h)

id

es

Method

SaveClear

Print

Quit

file: A:\WORK_BES

Method name: A:\RNA100

pe: General Ratio and Concentration

Units: μ g/ml

setup: VIEW

Background Correction: [No]

! device: None

Concentration: [Yes]

: range time: 0.50 sec

Peak Pick: [No]

abs 260.0 nm
abs 280.0 nm

260.0 nm

280.0 nm

Protein

Nucleic

Acid

260.0 nm

280.0 nm

 μ g/ml μ g/ml

0.1332 0.0801

1.6617

0.6018

0.0000 532.6299

0.5269 0.3118

1.6896

0.5918

0.0000 2107.5562

0.5868 0.3489

1.6822

0.5945

0.0000 2347.3457

0.6413 0.3719

1.7246

0.5799

0.0000 2565.3335

-0.3000 -0.3000

1.0000

1.0000

-0.0000 -1200.0000

✓ 0.5791 0.3421

1.6927

0.5908

0.0000 2316.2683

0.5624 0.3279

1.7151

0.5831

0.0000 2249.7939

1.0158 0.5580

1.8204

0.5493

0.0000 4063.1064

0.2683 0.1608

1.6678

0.5996

0.0000 1073.0630

0.5450 0.3118

1.7478

0.5722

0.0000 2180.0652

0.7140 0.4205

1.6980

0.5889

0.0000 2856.1829

0.5117 0.3033

1.6872

0.5927

0.0000 2046.7217

0.6982 0.4061

1.7192

0.5817

0.0000 2792.9055

0.6256 0.3685

1.6977

0.5890

0.0000 2502.4951

0.6685 0.3898

1.7152

0.5830

0.0000 2674.1338

0.4885 0.3012

1.6218

0.6166

0.0000 1954.0317

0.5343 0.3217

1.6609

0.6021

0.0000 2137.2446

0.5100 0.3112

1.6387

0.6102

0.0000 2040.1904

-0.3000 -0.3000

1.0000

1.0000

-0.0000 -1200.0000

0.5289 0.3211

1.6468

0.6072

0.0000 2115.4045

0.4389 0.2674

1.6415

0.6092

0.0000 1755.6592

0.5201 0.3133

1.6602

0.6023

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1.6689

0.5992

0.0000 2178.0122

0.5373 0.3215

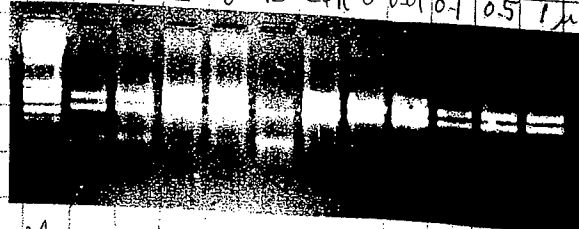
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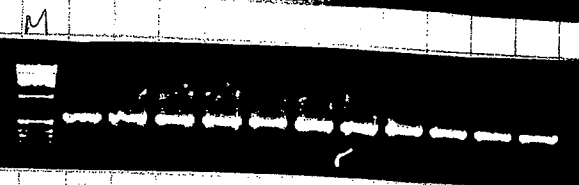
0.0000 2149.1904

H1299

Time course Dose



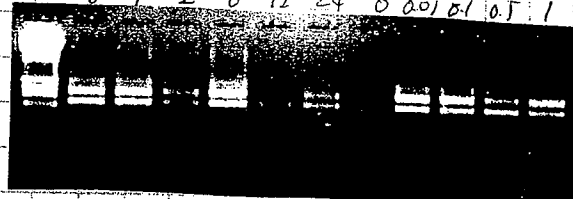
VEGF



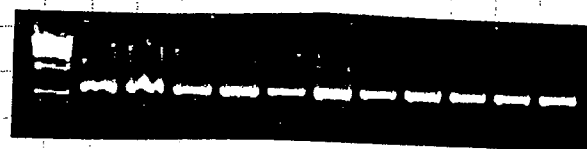
GAPDH

H460

Time course Dose



VEGF



GAPDH

ed & Understood by me,

Zoshi

Date

6/5/01

Invented by

J.S

Recorded by

J.S

Page No.

1/12/01

No. _____

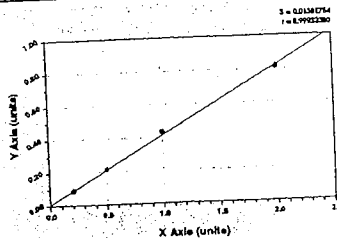
mple

Protein Concentration (Urea, BCA method); 010901

Albumin standard

Conc.(ug/ul)	0	0.2	0.5	1	2
OD	0	0.089	0.216	0.436	0.814

Conc.(ug/ul)	OD
0	0
0.2	0.089
0.5	0.216
1	0.436
2	0.814



PAGE = 10 /

1st Ab : caspase 3 (x500)
 caspase 9 (x500)
 PARP (x500)
 1 hour, rt

2nd Ab : Anti-mouse (PARP)
 Anti-Rabbit (caspase)
 1 hour, rt

Cell	H1299(24h)	50		
MZ(Um)	0	0.05	0.5	5
OD	2.184	2.257	1.893	1.763
OD(standard)	1.973	2.046	1.682	1.552
Protein(ug/ul)	4.82	5	4.11	3.79
50ug	10.37	10	12.17	13.19

Exposure : 5 sec, 5 min.

Re-probe : P21, BAX
 β-actin, P53

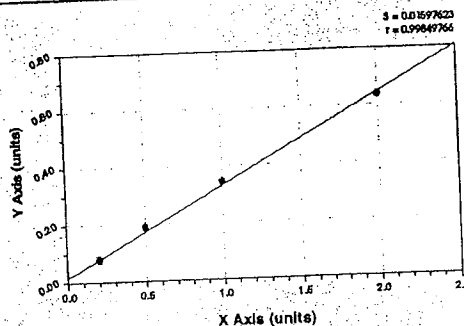
Cell	H460(24h)	50		
MZ(Um)	0	0.05	0.5	5
OD	2.536	2.49	2.279	2.381
OD(standard)	2.325	2.279	2.068	2.17
Protein(ug/ul)	5.69	5.57	5.06	5.31
50ug	8.79	8.98	9.88	9.42

Protein Concentration (Urea, BCA method); 011101

Albumin standard

Conc.(ug/ul)	0	0.2	0.5	1	2
OD	0	0.08	0.191	0.344	0.639

Conc.(ug/ul)	OD
0	0
0.2	0.08
0.5	0.191
1	0.344
2	0.639



Cell	A549(24h)	50	0.145			
Treatment	non-treat	UV(15J)	CDDP(10uM)	CDDP(50uM)	TAX(2uM)	TAX(10uM)
OD	0.952	0.693	0.992	0.815	0.944	0.823
OD(standarized)	0.807	0.548	0.847	0.67	0.799	0.678
Protein(ug/ul)	2.5	1.68	2.63	2.07	2.45	2.09
50ug	20	29.76	19.01	24.15	20.41	23.92

To Page No. _____

Inspected & Understood by me,

yoshi

Date

6/5/01

Invented by

J-S

Recorded by

J-S

Date

1/18/01

e No.

Sample: H460, H299

Treatment: MZ Dose (0, 0.2, 0.5, 1 μ M; 24h)

Lysate: Cell fractionation

Volume: 25 μ g

PAGE: 12.5%.

1st Ab: cytochrome C, COX IV (x500, x500) 1 hr r.t.

2nd Ab: Anti-mouse HRP (x2000)

Exposure: 15 sec and 5 min

Re-probe: PS3 & β -actinTo Page No.

I & Understood by me,

yoshi

Date

6/5/01

Invented by

J-S

Recorded by

J-S

Date

2/10/01

Page No. _____

Sample = same as p. 15.

Treatment: MR (0.1 μ M) \rightarrow time course (0, 12, 24, 48 h)
TAX (10 μ M), VAB (10 μ M)

Lysate = SDS-Urea PAGE 12.5-1. 50 μ g

First Ab: caspase 3, caspase 9 1 hour rt. (x 1000)

2nd Ab: Anti-Rabbit HRP (x 1000) 1 h rt.

Exposure: 15 sec

id & Understood by me,

joshi

Date

6/5/01

Invented by

J.S.

Recorded by

J.S.

Date

2/20/01

To Page No. _____

Page No.

Sample : H1299, H460

Treatment : MZ 0.5 μ M Time course (0, 30 min, 2, 6, 12, 24, 48 h)
MZ 24h Dose (0, 0.2 μ M, 0.5 μ M, 1 μ M)

Method : PI 70% Etoh fixation

See

FACS File (1)

To Page No.

Reviewed & Understood by me,

yoshi

Date

6/5/01

Invented by

J-S

Recorded by

J-S

Date

2/23

Page No. 18

Sample = same as p. 18.

PAGE = 12.5-1.

1st Ab: caspase 8, B:al (x 500)

2nd Ab: Anti-rabbit HRP (x 5000)

Exposure = 5min.

Protein Concentration (BF method); 022102

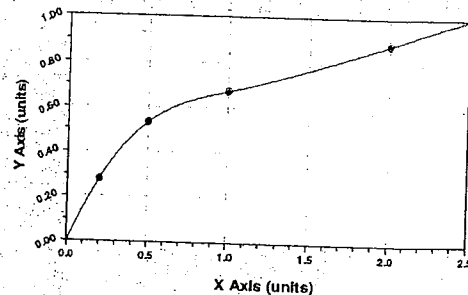
Albumin standard

Conc.(ug/ul)	0	0.2	0.5	1	2
OD	0	0.278	0.532	0.672	0.884
Conc.(ug/ul)	OD				
0	0				
0.2	0.278				
0.5	0.532				
1	0.672				
2	0.884				

Cell;H1299Cyto	Fraction-DTT	50	0.293	
Treatment	DMSO	MZ;0.2uM	MZ;0.2uM	MZ;1.0uM
OD	1.282	1.211	1.183	1.216
OD(standarized)	0.989	0.918	0.89	0.923
Protein(ug/ul)	2.46	2.14	2.03	2.17
50ug	20.33	23.36	24.63	23.04

Cell;H1299MT	Fraction-DTT			
Treatment	DMSO	MZ;0.2uM	MZ;0.2uM	MZ;1.0uM
OD	1.224	1.273	1.261	1.302
OD(standarized)	0.931	0.98	0.968	1.009
Protein(ug/ul)	2.2	2.42	2.36	2.55
50ug	22.73	20.66	21.19	19.61

Cell;H460Cyto	DMSO DTT				
Treatment	DMSO	MZ;0.2uM	MZ;0.2uM	MZ;1.0uM	CDDP;100uM
OD	1.23	1.197	1.269	1.243	
OD(standarized)	0.937	0.904	0.976	0.95	
Protein(ug/ul)	2.23	2.09	2.4	2.28	
50ug	22.42	23.92	20.83	21.93	22



PAGE = 12.5-1.

1st Ab: cytochrome C, COX IV (x 500)

2nd Ab: Anti-mouse HRP (x 2000)

Exposure = 5min.

yuzhi

6/5/01

J.S

J.S

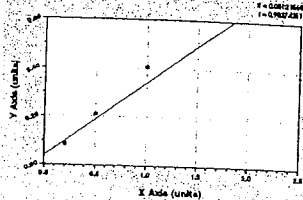
2/26/01

No. Sample

Protein Concentration (BCA method); 021501

Protein standard

Conc. (ug/ul)	0	0.2	0.5	1	2
OD	0	0.088	0.213	0.409	0.61
0	0				
0.2	0.088				
0.5	0.213				
1	0.409				
2	0.61				



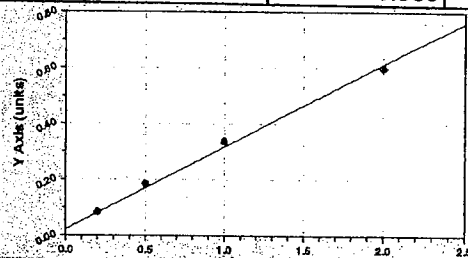
L1460		SDS-DTT		50	0.114
Protein		0	UV;30min	MZ;30min	MZ;2h
(standardized)		0.585	0.602	0.615	0.634
Protein(ug/ul)		0.471	0.488	0.501	0.52
g		1.42	1.48	1.52	1.58
L1460		35.21	33.78	32.89	31.65

Protein	SDS-DTT	50	0.364
Protein	MZ;6h	MZ;12h	MZ;24h
(standardized)	0.782	0.908	0.947
Protein(ug/ul)	0.418	0.544	0.583
g	1.41	1.87	2.01
	35.46	26.74	24.88

Protein Concentration (BCA method); 022101

Protein standard

Conc. (ug/ul)	0	0.2	0.5	1	2
OD	0	0.086	0.185	0.339	0.602
0	0				
0.2	0.086				
0.5	0.185				
1	0.339				
2	0.602				



99	SDS-DTT		50	0.227		
n	DMSO;6h	MZ;0.2uM,6h	MZ;0.3uM,6h	MZ;0.4uM,6h	MZ;0.5uM,6h	MZ;1.0uM,6h
larized)	0.997	0.938	0.876	0.971	0.932	0.929
g/ul)	0.77	0.711	0.649	0.744	0.705	0.702
	2.52	2.32	2.11	2.43	2.3	2.29
	19.84	21.55	23.7	20.58	21.74	21.83

SDS-DTT		20.55	20.7	20.58	21.74	21.83	
Protein	DMSO;6h	MZ;0.2uM,6h	MZ;0.3uM,6h	MZ;0.4uM,6h	MZ;0.5uM,6h	MZ;1.0uM,6h	MZ;6h'
(standardized)	0.872	0.82	0.699	0.717	0.738	0.788	
Protein(ug/ul)	0.645	0.593	0.472	0.49	0.511	0.561	
g	2.1	1.92	1.52	1.58	1.65	1.82	
	23.81	26.04	32.89	31.65	30.3	27.47	30

PAGE: 104.

TBS system

Unpublished by
yoshi

Date
6/5/01

Reviewed by
J.S.

J.S.

3/2-6

Page No. 24

Page No. 20

1st Ab = (P) - Jun, (P) - ATF2 (3/2)
 (P) - P38, (P) - P42/p44 ERK (3/3)
 (P) - Bad, (P) - AKT (3/6)

overnight, 4°C

2nd Ab = Anti Rabbit HRP (x5000)

Exposure = 30 sec - 30 min

FACS

Project No. 5

Book No. 2

Sample: H460

Treatment: { DMSO (24h, 48h, 72h)
 MZ 0.2 μ M (24h, 48h, 72h)
 MZ 0.5 μ M (24h, 48h, 72h)
 MZ 1.0 μ M (24h, 48h, 72h)
 CDOP 50 μ M (24h, 48h, 72h)

See FACS File (2)

3/1/01

FACS

Project No. 5

Book No. 2

Sample = H460, H460. A549

Treatment: DMSO (12h, 24h, 48h)
 MZ 0.2 μ M (12h, 24h, 48h)
 MZ 0.5 μ M (12h, 24h, 48h)
 MZ 1.0 μ M (12h, 24h, 48h)

See FACS File (3)

3/16/01

Investigated & Understood by me,

Yoshi

Date

6/5/01

Invented by

J.S

Recorded by

J-S

Date

3/2-6

Page No.

Page No. _____

Re-probe

Sample: Same as P21. (H460, H299 dose & Time)

1st Ab

HIF-1 α	(x250)	Cyclin G1	(x200)
P21	(x500)	Cyclin A	(x500)
P27	(x200)		
GRP 78	(x200)		
GADD 153	(x200)		
BAX	(x500)		
Chk-1	(x200)		
Chk-2	(x200)	1 hour r.t.	

2nd Ab

Anti-mouse HRP	(HIF-1 α , P21, ^{Cyclin A} Chk-1)	x2000
Anti-Rabbit HRP	(BAX, Chk-2, GADD153)	x5000
Anti-goat HRP	(P27, GRP78) _{Cyclin G1}	x10000

1 hour r.t.

Exposure (30 s, 3 min, 30 min)

yoshi

6/5/01

Invented by J-S
Responsible J-S

Date

3/19

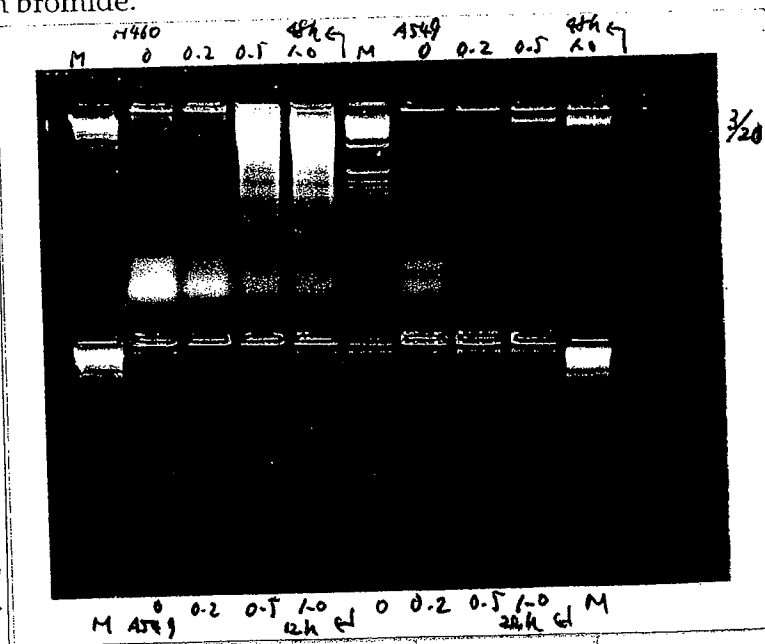
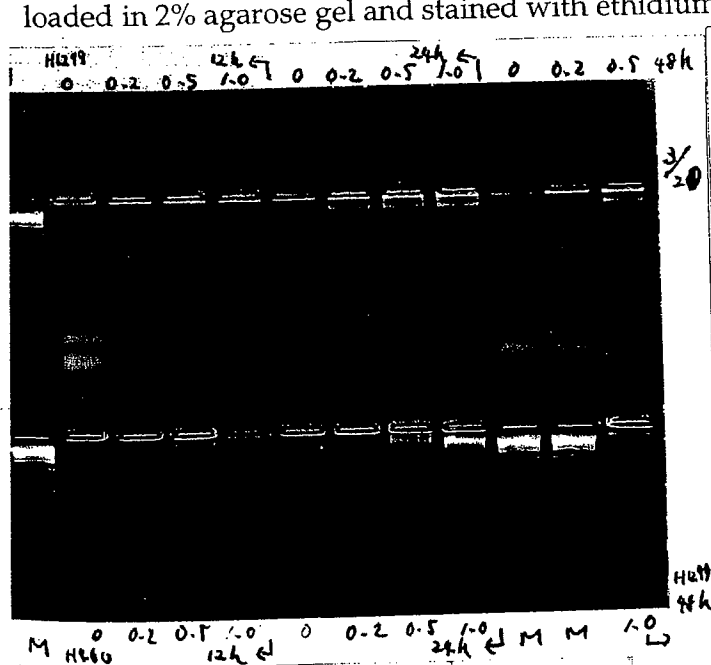
e No. _____

Cell: H460, A549, H1299

Treatment: MZ dose: 0, 0.2, 0.5, 1 μ M Time: 12h, 24h, 48h

DNA fragmentation assay

The nucleosomal DNA degradation was analyzed as described previously with modification. Briefly, 5×10^5 cells of each cell line were seeded in 100-mm culture dish and allowed to grow for 24 hours. The medium was then replaced with medium containing either DMSO or several doses of MZ. After 12, 24, 48 hours of incubation, both floating and attached cells were harvested and washed in cold PBS. The cell pellets were stored at -80°C until use. The pellets were lysed in 100 mm^3 of the lysis buffer (10 mM Tris (pH 7.4), 10mM EDTA (pH 8.0), 0.5% Triton X-100) and incubated for 10 min at 4°C . After centrifugation, the supernatants were incubated with $200 \mu\text{g}/\text{ml}$ of RNase A for 1 hour at 37°C . The supernatants were then incubated with $200 \mu\text{g}/\text{ml}$ of Proteinase K for 30 min at 50°C . After the incubation, DNA fragments were precipitated with 0.5 M of NaCl and 50% of isopropanol. The sample was loaded in 2% agarose gel and stained with ethidium bromide.



Described in: [illegible]

Date

J.S

Date

J.S

3/20

Zoshi

6/5/01

No. SampleProtein Concentration (BF method); 030701
Albumin standard

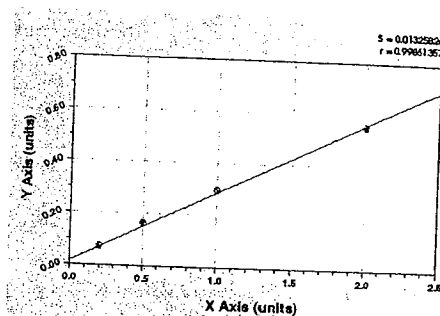
MR: 0.5uM

Conc.(ug/ul)	0	0.2	0.5	1	2
OD	0	0.368	0.658	0.804	0.924
Conc.(ug/ul)	OD				
0	0				
0.2	0.368				
0.5	0.658				
1	0.804				
2	0.924				

Cell;H1299Cyto	Fraction-DTT	50	0.312	
Treatment	0h	6h	12h	24h
OD	1.303	1.312	1.347	1.316
OD(standarized)	0.991	1	1.035	1.004
Protein(ug/ul)	2.5	2.5	2.5	2.5
50ug	20	20	20	20
Cell;H460Cyto	Urea-DTT			
Treatment	0h	6h	12h	24h
OD	1.255	1.195	1.218	1.215
OD(standarized)	0.943	0.883	0.906	0.903
Protein(ug/ul)	2.18	1.62	1.78	1.78
50ug	22.94	30.86	28.09	28.09

Concentration (BCA method); 032201

standard	0.092
ug/ul)	OD
0	0
0.2	0.074
0.5	0.164
1	0.297
2	0.554



299, 48h	Urea-DTT		X Axis (units)			
	50		0.139			
ant	DMSO+P53(1moi)	MZ;0.1uM+P53(1moi)	MZ;0.2uM+P53(1moi)	MZ;0.2uM+GFP(1moi)	MZ;0.5uM	P53(10moi)
	1.232	1.089	0.957	1.326	0.995	0.486
darized)	1.093	0.95	0.818	1.187	0.856	0.347
ug/ul)	3.95	3.42	2.94	4.29	3.08	1.21
	12.66	14.62	17.01	11.66	16.23	41.32

60, 48h	Urea-DTT					
ant	DMSO+P53(1moi)	MZ;0.1uM+P53(1moi)	MZ;0.2uM+P53(1moi)	MZ;0.2uM+GFP(1moi)	MZ;0.5uM	P53(10moi)
	1.728	1.316	1.349	1.177	0.718	1.318
darized)	1.589	1.177	1.21	1.038	0.579	1.179
ug/ul)	5.77	4.26	4.38	3.75	2.06	4.26
	8.67	11.74	11.42	13.33	24.27	11.74

To Page No. 25

Reviewed by

Date

2/21/02

Initiated by

J.S

Recorded by

J.S

3/29-3/01

ie No. 29

1st Ab : Cytochrome C (x500) → Fraction (3/7) }
 Caspase 3 (x500) → Urea (3/22) } (3/29)

~~Re-probe : β -actin & P53 (3/31)~~

PARP (x1000) } Urea (3/22) (3/30)
 HIF1- α (x200) }
 P53 + β -actin

Re-probe : β -actin & P53

To Page No. _____

Read & Understood by me,

Joshi

Date

3/21/0

Invented by

J.S

Recorded by

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Date

3/24/3

Page No. _____

Sample : Fraction sample H1299, H460 → (3/7)
 Urea-SDS sample H1299, H460 →

Protein Concentration (BCA method); 022701
 Albumin standard

Conc.(ug/ul)	0	0.2	0.5	1	2
OD	0	0.094	0.208	0.336	0.626
Conc.(ug/ul)	OD				
0	0				
0.2	0.094				
0.5	0.208				
1	0.336				
2	0.626				

Cell; H1299 48h Urea-DTT

Treatment	DMSO	MZ; 0.2uM	MZ; 0.5uM	MZ; 1.0uM
OD		50	0.236	
OD(standardized)	1.957	1.702	1.153	1.231
Protein(ug/ul)	1.721	1.466	0.917	0.995
50ug	5.57	4.73	2.93	3.18
	8.98	10.57	17.06	15.72

Cell; H460 48h Urea-DTT

Treatment	DMSO	MZ; 0.2uM	MZ; 0.5uM	MZ; 1.0uM
OD				
OD(standardized)	1.752	1.554	0.928	0.785
Protein(ug/ul)	1.516	1.318	0.692	0.549
50ug	4.9	4.25	2.19	1.71
	10.2	11.76	22.83	29.24

Ab : Cytochrome C (x1000) → (3/7)
 Caspase 3 (x500)
 Caspase 9 (x500) } (2/3)
 Caspase 8 (x500) } 4/3

probe β-actin → Cytochrome C, (Caspase 3)
 P53 → Caspase 9
 P21 → Caspase 8 } 4/4

Underlined

roshi

2/21/02

J.S

J.S

To Page No. _____

Date

4/3-4/01

e No. 27

Cyto	Fraction-DTT		50	0.018
	DMSO	MZ;0.2uM	MZ;0.5uM	MZ;1.0uM
	1.017	1	1	1.035
arized)	0.999	0.982	0.982	1.017
/ul)	1.98	1.89	1.89	2.07
	25.25	26.46	26.46	24.15

MT	Fraction-DTT		50	0.018
	DMSO	MZ;0.2uM	MZ;0.5uM	MZ;1.0uM
	1.1	1.12	1.01	1.01
arized)	1.082	1.102	0.992	0.992
/ul)	2.4	2.49	1.94	1.94
	20.83	20.08	25.77	25.77

UUC	Fraction-DTT		50	0.018
	DMSO	MZ;0.2uM	MZ;0.5uM	MZ;1.0uM
	1.242	1.229	1.308	1.256
arized)	1.224	1.211	1.29	1.238
/ul)	2.99	2.95	3.21	3.04
	16.72	16.95	15.58	16.45

Cyto	Fraction-DTT		50	0.018			
	DMSO	MZ;0.2uM	MZ;0.2uM vnb	vnb		MZ; 0.2uM, 0h	MZ; 0.2uM, 1h
	0.947	0.969	0.93	0.925		0.938	0.91
rized)	0.929	0.951	0.912	0.907		0.92	0.9
ul)	1.63	1.73	1.54	1.52		1.58	1.4
	30.67	28.9	32.47	32.89		31.65	33.5

4T	Fraction-DTT		50.07	28.9	32.47	32.89	31.65	33.0
	DMSO	MZ;0.2uM	MZ;0.2uM vnb	vnb		MZ; 0.2uM, 0h	MZ; 0.2uM, 1h	
	0.9	0.975	1.007	0.967		0.913	0.913	
arized)	0.882	0.957	0.989	0.949		0.895	0.895	
ul)	1.41	1.76	1.93	1.72		1.47	1.47	
	35.46	28.41	25.91	29.07		34.01	34.01	
IUC	Fraction-DTT		50.07	28.9	32.47	32.89	31.65	33.0

IUC	Fraction-DTT							
	DMSO	MZ;0.2uM	MZ;0.2uM vnb	vnb	MZ; 0.2uM, 0h	MZ; 0.2uM,		
	1.237	1.242	1.221	1.232	1.159	1.2		
ized)	1.219	1.224	1.203	1.214	1.141	1.2		
ul)	2.97	2.99	2.92	2.96	2.67	2.		
	16.84	16.72	17.12	16.89	18.73	16.		

t: Ab : cytochrome C (x1000) → (H460 Frac - MZ+UNB - cytosolic
H1299 Frac - MZ+P53 - cytosolic)

Caspase 3, caspase 9 (x500) → ARE9 Urea-SDS

To Page No.

yoshi

2/21/02

J.S

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4/14

Page No.

Sample : (4/10)

1st Ab : BAX (x1000) → (H460 Frac-MZ+VNB - mitochondrial
H1299 Frac-MZ+P53 - mitochondrial)

Caspase 3, caspase 9 H460 Urea-DTT + P53 + MZ

Re-probe : P53 (x1000) → A549 Urea-SDS, H460-HitoF

P21 (x1000) → A549 Urea-SDS

B-actin → H1299 - cyto F, H460 - cyto F, H460-Urea-DTT P53+MZ

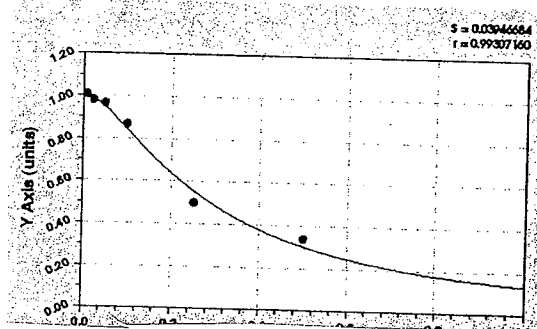
XTT assay

Project 5/
Book No. 2

41901 XTT-48h1299 MZ-dose

blank	0(uM)	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
0.196	1.176	1.146	1.137	1.154	1.024	0.55	0.381	0.363	0.341	0.323	0.352
0.19	1.138	1.168	1.19	1.156	1.037	0.569	0.41	0.361	0.357	0.354	0.341
0.19	1.201	1.193	1.16	1.101	0.977	0.566	0.412	0.369	0.372	0.348	0.308
0.193	1.187	1.185	1.108	1.111	1.066	0.592	0.398	0.419	0.344	0.363	0.361
	1.231	1.186	1.089	1.07	1.008	0.6	0.426	0.368	0.354	0.345	0.353
	1.135	1.188	1.141	1.12	1.009	0.572	0.387	0.356	0.333	0.319	0.363
	1.121	1.185	1.135	1.159	1.01	0.607	0.402	0.343	0.355	0.323	0.323
	1.06	1.123	1.098	1.103	0.957	0.595	0.37	0.353	0.332	0.326	0.308
average	1.1561	1.1718	1.1323	1.122	1.011	0.58138	0.3983	0.3665	0.3485	0.337625	0.338625
ratio	1	1.01	0.98	0.97	0.87	0.5	0.34	0.32	0.3	0.29	0.29

0	1
0.01	1.01
0.025	0.98
0.05	0.97
0.1	0.87
0.25	0.5
0.5	0.34
1	0.32
2.5	0.3
5	0.29



IC20	0.12
IC50	0.288
IC80	0.72

Used & Understood by me,

Zozli

Date

2/21/02

Invented by

J. S

Recorded by

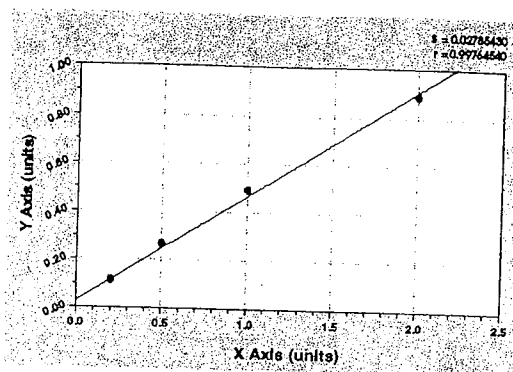
J. S

Date

4/19/01

Project No. 5Book No. 2TITLE Western Blotage No. Sample = H460 ①Concentration (BCA method); 042101
n standard

ug/ul)	OD
0	0
0.2	0.116
0.5	0.267
1	0.496
2	0.888



549	Urea-DTT		50	0.092 x5				
ent	MZ; 5uM, 0h	MZ; 5uM, 12h	MZ; 5uM, 24h	MZ; 5uM, 48h	DMSO; 48h	1uM; 48h	5uM; 48h	10uM; 48h
	0.573	0.64	0.628	0.446	0.536	0.448	0.415	0.44
ndarized)	0.481	0.548	0.536	0.354	0.444	0.356	0.323	0.348
(ug/ul)	1.03	1.18	1.16	0.74	0.95	0.75	0.67	0.73
(ug/ul) True	5.15	5.9	5.8	3.7	4.75	3.75	3.35	3.65
	9.71	8.47	8.62	13.51	10.53	13.33	14.93	13.7

160 ①	SDS-DTT		50	0.334 x5					
ent	UV;2h	CDDP(50uM)	MZ;0.5uM, 0h	12h	24h	48h	DMSO;48h	MZ;0.2uM	M
	0.726	0.67	0.752	0.644	0.549	0.658	0.918	0.909	
ndarized)	0.392	0.336	0.418	0.31	0.215	0.324	0.584	0.575	
(ug/ul)	0.83	0.7	0.89	0.64	0.42	0.67	1.26	1.24	
(ug/ul) True	4.15	3.5	4.45	3.2	2.1	3.35	6.3	6.2	
	12.05	14.29	11.24	15.63	23.81	14.93	7.94	8.06	

99 SDS-DTT		50 0.147 x5								
it	Co(100uM)	Aniso;50nM	UV;2h	MZ; 0.5uM	12h	24h	48h	DMSO; 0.2uM	0.5uM	1uM
	0.394	0.39	0.453	0.297	0.308	0.316	0.407	0.761	0.663	0.525
lar	0.247	0.243	0.306	0.15	0.161	0.169	0.26	0.614	0.516	0.378
g/u	0.84	0.82	1.05	0.48	0.52	0.55	0.88	2.17	1.81	1.31
g/u	4.2	4.1	5.25	2.4	2.6	2.75	4.4	10.85	9.05	6.55
	11.9	12.2	9.52	20.83	19.23	18.18	11.36	4.61	5.52	7.63
										9.09

9①SDS-DTT											
50 0.147 x5											
it	Co(100uM)	Aniso;50nM	UV;2h	CDDP(50	MZ; 0h, 12h	24h	48h	DMSO	0.5uM	1uM	
	0.427	0.374	0.41	0.241	0.366	0.3	0.303	0.341	0.442	0.36	0.321
lar	0.28	0.227	0.263	0.094	0.219	0.153	0.156	0.194	0.295	0.213	0.174
g/l	0.96	0.76	0.89	0.28	0.73	0.49	0.5	0.64	1.01	0.71	0.57
g/l	4.8	3.8	4.45	1.4	3.65	2.45	2.5	3.2	5.05	3.55	2.85
	10.42	13.16	11.24	35.71	13.7	20.41	20	15.63	9.9	14.08	17.54

② SDS-DTT											
50 0.147 x5											
t	Co(100uM)	Aniso; 50nM	UV; 2h	MZ; 0.5uM	12h	24h	48h	DMSO; 0.2uM	0.5uM	1uM	N
	0.576	0.393	0.416	0.401	0.263	0.297	0.415	0.726	0.79	0.539	0.434
ar	0.429	0.246	0.269	0.254	0.116	0.15	0.268	0.579	0.643	0.392	0.287
y/u	1.5	0.83	0.92	0.86	0.36	0.48	0.88	2.04	2.27	1.36	0.98
y/u	7.5	4.15	4.6	4.3	1.8	2.4	4.4	10.2	11.35	6.8	4.9
	6.67	12.05	10.87	11.63	27.78	20.83	11.36	4.9	4.41	7.35	10.2

sed & Understood by me,

yoshi

Date

2/21/02

Invented by

J.S

Recorded by

J.S

Date

4/26-27

0.31

Page No. _____ Growth inhibition assay

Design

day 0

Seed cells

1000/well (H460, H1299)

2000/well (A549)

day 1

Treatment MR (0 ~ 10 μ M)

with or without inhibitory agents

1) DMSO - control

2) PDTC - JNK inhibitor

3) SB202190

4) SB203580 } p38 inhibitor

5) LY294002 - ~~PI3K~~ PI3K inhibitor

6) U012 - MEK inhibitor

day 3

XTT

A549

data 6/6/97 A549 DMSO/PDTC

	1	2	3	4	5	6	7	8	9	10	11	12
		1.301	1.319	1.329	1.266	1.218	0.884	0.778	0.681	0.69	0.714	0.66
		1.278	1.328	1.275	1.264	1.184	0.888	0.784	0.683	0.717	0.665	0.642
		1.348	1.27	1.264	1.294	1.231	0.9	0.782	0.72	0.699	0.694	0.658
		1.308	1.301	1.235	1.279	1.235	0.977	0.907	0.677	0.652	0.657	0.607
202		1.301	1.294	1.179	1.202	1.163	0.821	0.766	0.71	0.695	0.673	0.661
201		0.276	0.29	0.295	0.272	0.297	0.284	0.323	0.301	0.32	0.316	0.34
208		0.21	0.211	0.21	0.206	0.211	0.211	0.214	0.21	0.213	0.216	0.222
216		0.223	0.221	0.225	0.215	0.219	0.219	0.218	0.219	0.22	0.221	0.225
575												

MZ(μ M)

(%)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
0	1.09425	1.11225	1.12225	1.05925	1.01125	0.67725	0.57125	0.47425	0.48325	0.50725	0.45325
08	1.07125	1.12125	1.06825	1.05725	0.97725	0.68125	0.57725	0.47625	0.51025	0.45825	0.43525
08	1.14125	1.06325	1.05725	1.08725	1.02425	0.69325	0.57525	0.51325	0.49225	0.48725	0.45125
24	1.10125	1.09425	1.02825	1.07225	1.02825	0.77025	0.70025	0.47025	0.44525	0.45025	0.40025
M)											
0	1.09425	1.08725	0.97225	0.99525	0.95625	0.61425	0.55925	0.50325	0.48825	0.46625	0.45425
1	0.06925	0.08325	0.08825	0.06525	0.09025	0.07725	0.11625	0.09425	0.11325	0.10925	0.13325
25	0.00325	0.00425	0.00325	0.0007	0.00425	0.00425	0.00725	0.00325	0.00625	0.00925	0.01525
20	0.01625	0.01425	0.01825	0.00825	0.01225	0.01225	0.01125	0.01225	0.01325	0.01425	0.01825

MZ(μ M)

(%)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
0	1	1.016	1.026	0.968	0.924	0.619	0.522	0.433	0.442	0.464	0.414
8	0.979	1.025	0.976	0.966	0.893	0.623	0.528	0.435	0.466	0.419	0.398
8	1.043	0.972	0.966	0.994	0.936	0.634	0.526	0.469	0.45	0.445	0.412
4	1.006	1	0.94	0.98	0.94	0.704	0.64	0.43	0.407	0.411	0.366
4)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
0	1	0.994	0.889	0.91	0.874	0.561	0.511	0.46	0.446	0.426	0.415
1	0.063	0.076	0.081	0.06	0.082	0.071	0.106	0.086	0.103	0.1	0.122
5	0.003	0.004	0.003	0.001	0.004	0.004	0.007	0.003	0.006	0.008	0.014
2	0.015	0.013	0.017	0.008	0.011	0.011	0.01	0.011	0.012	0.013	0.017

Read & Understood by me

Yoshi

Date

2/21/02

Invented by

J-S

Recorded by

J-S

Date

Jan/6

XTT assay

Project 1

Book No. 2

3

Page No. 38

H460

Row c: 6/6/97 H460 DMSO/PDTC

	2	3	4	5	6	7	8	9	10	11	12
	1.738	1.468	1.558	1.539	1.513	0.614	0.495	0.385	0.428	0.414	0.369
	1.591	1.526	1.456	1.467	1.408	0.564	0.458	0.389	0.426	0.422	0.377
	1.591	1.596	1.579	1.585	1.51	0.571	0.49	0.4	0.418	0.409	0.369
	1.495	1.511	1.579	1.505	1.529	0.604	0.475	0.408	0.397	0.422	0.372
	1.554	1.555	1.611	1.557	1.51	0.636	0.482	0.397	0.42	0.429	0.369
	0.211	0.214	0.209	0.209	0.208	0.212	0.207	0.212	0.208	0.216	0.216
	0.225	0.22	0.221	0.214	0.218	0.214	0.218	0.213	0.219	0.216	0.216
	0.229	0.23	0.228	0.227	0.226	0.226	0.225	0.224	0.223	0.218	0.222

MZ(uM)

0(%)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
0	1.516	1.246	1.336	1.317	1.291	0.392	0.273	0.163	0.206	0.192	0.147
0.008	1.369	1.304	1.234	1.245	1.186	0.342	0.236	0.167	0.204	0.2	0.155
0.08	1.369	1.374	1.357	1.363	1.288	0.349	0.268	0.178	0.196	0.187	0.147
0.4	1.273	1.289	1.357	1.283	1.307	0.382	0.253	0.186	0.175	0.2	0.15
SI (uM)											
0	1.332	1.333	1.389	1.335	1.288	0.414	0.26	0.175	0.198	0.207	0.147
1	0	0	0	0	0	0	0	0	0	0	0
25	0.003	0	0	0	0	0	0	0	0	0	0
100	0.007	0.008	0.006	0.005	0.004	0.004	0.003	0.002	0.001	0	0

MZ(uM)

0(%)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
0	1	0.822	0.881	0.869	0.852	0.259	0.18	0.108	0.136	0.127	0.097
0.08	0.903	0.86	0.814	0.821	0.782	0.226	0.156	0.11	0.135	0.132	0.102
0.08	0.903	0.906	0.895	0.899	0.85	0.23	0.177	0.117	0.129	0.123	0.097
0.4	0.84	0.85	0.895	0.846	0.862	0.252	0.167	0.123	0.115	0.132	0.099
M											
0	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
0	1	1.001	1.043	1.002	0.967	0.311	0.195	0.131	0.149	0.155	0.11
1	0	0	0	0	0	0	0	0	0	0	0
5	0.002	0	0	0	0	0	0	0	0	0	0
0	0.005	0.006	0.005	0.004	0.003	0.003	0.002	0.002	0.001	0	0

Assayed & Up-dated by

goshi

2/1/02

Recorded by

J.S

J.S

Jun/6

40

XTT assay

Project No. _____

Book No. 2

41

Page No. _____ To investigate MZ activity to pancreas cancer cells.

Design : day 0 day 1 day 3
Seed cells. Treatment with XTT assay
2000/well DMSO or MZ (0-10 μ M)

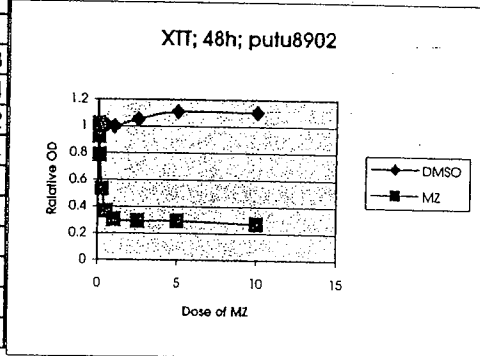
Sample : Putu 8902, Putu 8988T, Putu 8988S

Row data 6/7/01 PUTU8902

Row data 6/7/01 PUTU8902

1	2	3	4	5	6	7	8	9	10	11	12
0.169	0.543	0.551	0.518	0.547	0.583	0.562	0.555	0.562	0.599	0.613	0.603
0.165	0.55	0.547	0.555	0.561	0.567	0.545	0.573	0.544	0.582	0.577	0.579
0.17	0.543	0.547	0.558	0.567	0.515	0.526	0.528	0.534	0.552	0.576	0.581
0.179	0.541	0.542	0.556	0.494	0.529	0.523	0.533	0.535	0.521	0.575	0.57
	0.54	0.546	0.538	0.487	0.43	0.357	0.297	0.28	0.273	0.281	0.273
	0.547	0.543	0.548	0.492	0.457	0.356	0.308	0.275	0.279	0.278	0.265
	0.545	0.557	0.542	0.534	0.48	0.379	0.313	0.288	0.285	0.282	0.267
0.17075	0.542	0.555	0.537	0.541	0.485	0.382	0.305	0.291	0.282	0.279	0.28
DMSO(%)	0	0.0001	0.00025	0.0005	0.001	0.0025	0.005	0.01	0.025	0.05	0.1
	0.37225	0.38025	0.34725	0.37625	0.41225	0.39125	0.38425	0.39125	0.42825	0.44225	0.43225
	0.37925	0.37625	0.38425	0.39025	0.39625	0.37425	0.40225	0.37325	0.41125	0.40625	0.40825
	0.37225	0.37625	0.38725	0.39625	0.34425	0.35525	0.35725	0.36325	0.38125	0.40525	0.41025
	0.37025	0.37125	0.38525	0.32325	0.35825	0.35225	0.36225	0.36425	0.35025	0.40425	0.39925
AVERAG	0.3735	0.376	0.376	0.3715	0.37775	0.36825	0.3765	0.373	0.39275	0.4145	0.4125
RATIO	1	1.007	1.007	0.995	1.011	0.986	1.008	0.999	1.052	1.11	1.104
MZ(uM)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10
	0.36925	0.37525	0.36725	0.31625	0.25925	0.18625	0.12625	0.10925	0.10225	0.11025	0.10225
	0.37625	0.37225	0.37725	0.32125	0.28625	0.18525	0.13725	0.10425	0.10825	0.10725	0.09425
	0.37425	0.38625	0.37125	0.36325	0.30925	0.20825	0.14225	0.11725	0.11425	0.11125	0.09625
	0.37125	0.38425	0.36625	0.37025	0.31425	0.21125	0.13425	0.12025	0.11125	0.10825	0.10925
AVERAG	0.37275	0.3795	0.3705	0.34275	0.29225	0.19775	0.135	0.11275	0.109	0.10925	0.1005
RATIO	1	1.018	0.994	0.92	0.784	0.531	0.362	0.302	0.292	0.293	0.27
	DMSO	MZ									
0	1	1									
0.01	1.007	1.018									
0.025	1.007	0.994									
0.05	0.995	0.92									
0.1	1.011	0.784									
0.25	0.986	0.531									
0.5	1.008	0.362									
1	0.999	0.302									
2.5	1.052	0.292									
5	1.11	0.293									
10	1.104	0.27									

DMSO	MZ
0	1
0.01	1.007
0.025	1.007
0.05	0.995
0.1	1.011
0.25	0.986
0.5	1.008
1	0.999
2.5	1.052
5	1.11
10	1.104



MZ	
0	1
0.01	1.018
0.025	0.994
0.05	0.92
0.1	0.784
0.25	0.531
0.5	0.362
1	0.302
2.5	0.292
5	0.293
10	0.27

IC20	0.09
IC50	0.33
IC80	1.25

Seed 3. Understood by me.

zorch

Date

2/21/02

Invented by

J.S

Recorded by

J.S

Date

Jun 7

To Page No.

XTT assay

Page No.

Design: Same as page 41

Sample: Putu 8988†, Putu 89885, MCF-7, Putu 8902

data 6/16/01 PUTU8988† PUTU8902												
1	2	3	4	5	6	7	8	9	10	11	12	
0.23	0.555	0.574	0.575	0.582	0.579	0.526	0.429	0.387	0.359	0.336	0.334	0.4275
0.224	0.573	0.571	0.584	0.596	0.587	0.529	0.437	0.385	0.352	0.346	0.334	0.4485
0.227	0.588	0.576	0.603	0.607	0.583	0.521	0.441	0.379	0.371	0.343	0.335	0.4105
0.227	0.577	0.601	0.586	0.587	0.572	0.552	0.434	0.388	0.355	0.348	0.332	0.4145
	0.753	0.733	0.739	0.752	0.711	0.548	0.439	0.369	0.381	0.368	0.362	0.42525
	0.71	0.754	0.743	0.732	0.688	0.537	0.424	0.377	0.373	0.367	0.355	0.74
	0.7	0.745	0.753	0.733	0.689	0.556	0.427	0.366	0.37	0.377	0.358	
0.227	0.689	0.735	0.744	0.71	0.673	0.537	0.426	0.363	0.361	0.364	0.352	10
U8988†												0.5745
(UM)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10	0.6185
	0.328	0.347	0.348	0.355	0.352	0.299	0.202	0.16	0.132	0.109	0.107	0.6095
	0.346	0.344	0.357	0.369	0.36	0.302	0.21	0.158	0.125	0.119	0.107	0.6075
	0.361	0.349	0.376	0.38	0.356	0.294	0.214	0.152	0.144	0.116	0.108	0.6025
	0.35	0.374	0.359	0.36	0.345	0.325	0.207	0.161	0.128	0.121	0.105	0.74
ERAG	0.34625	0.3535	0.36	0.366	0.35325	0.305	0.20825	0.15775	0.13225	0.11625	0.10675	
PIO	1	1.02	1.04	1.06	1.02	0.88	0.6	0.46	0.38	0.34	0.31	
U8902												
(UM)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10	
	0.526	0.506	0.512	0.525	0.484	0.321	0.212	0.142	0.154	0.141	0.135	
	0.483	0.527	0.516	0.505	0.461	0.31	0.197	0.15	0.146	0.14	0.128	
	0.473	0.518	0.526	0.506	0.462	0.329	0.2	0.139	0.143	0.15	0.131	
	0.462	0.508	0.517	0.483	0.446	0.31	0.199	0.136	0.134	0.137	0.125	
ERAG	0.486	0.51475	0.51775	0.50475	0.46325	0.3175	0.202	0.14175	0.14425	0.142	0.12975	
PIO	1	1.06	1.07	1.04	0.95	0.65	0.42	0.29	0.3	0.29	0.27	
w data 6/16/01 PUTU81885 MCF-7												
1	2	3	4	5	6	7	8	9	10	11	12	
0.232	0.805	0.775	0.793	0.778	0.701	0.695	0.622	0.584	0.606	0.639	0.657	
0.227	0.813	0.765	0.75	0.803	0.736	0.718	0.62	0.588	0.603	0.63	0.678	
0.228	0.805	0.78	0.801	0.805	0.75	0.72	0.599	0.579	0.651	0.68	0.64	
0.231	0.802	0.809	0.818	0.823	0.777	0.697	0.568	0.616	0.659	0.638	0.644	
	1.05	1.1	1.02	1.031	0.813	0.834	0.803	0.781	0.852	0.844	0.804	
	1.026	1.03	1.05	1.047	1.045	0.897	0.86	0.812	0.808	0.835	0.848	
	1.042	1.053	1.068	1.027	1.067	0.891	0.867	0.864	0.838	0.814	0.839	
0.2295	1.059	1.07	1.037	1.054	1.063	0.848	0.797	0.808	0.777	0.796	0.837	
JU81885												
(UM)	0	0.01	0.025	0.05	0.1	0.25	0.5	1	2.5	5	10	

Witnessed & Underwritten by

Zozhi

Date

2/21/02

J.S.

Witnessed by

J.S.

June/16/01

Page No.

m Page No. To quantify the efficacy of tubulin polymerization/depolymerization by MR,
we developed modified tubulin polymerization assay.

Design

day 0	day 1	
Seed cells in 12 well (5×10^4 /well/2ml) $\times 3$	Treatment with Lysis with	
	DMSO	
	MR (0.5 μ M)	
	NCE (0.5 μ M)	for 5 min
	TAX (10 nM)	for 3 hours
		→ Assay. (Immunoblot with α -tubulin Ab)

⇒ All treatment showed soluble tubulin expression in blot!

Immunostaining = α -tubulin
Sample: H460, A549, H1299

Design

day 0	→ day 1	→ day 2
1×10^4 cells/well (2 x chamber slide)	Treatment DMSO MR = 0.5 μ M NCE = 2.5 μ M TAX = 10 nM	Stain

- 1) Remove medium and wash with cold PBS
- 2) Fix cells in 4% Paraformaldehyde for 10 min at 4°C
- 3) Wash in cold PBS
- 4) Permeabilizes cells with 1ml solution containing Triton-X 100 0.1% and Sodium Citrate 0.1% for 2 min.
- 5) Wash in cold PBS twice
- 6) Blocking for 20 min
- 7) do same as 5)
- 8) Priming Ab reaction for 1 hour
- 9) do same as 5)
- 10) Secondary Ab reaction for 1 hour (with DAPI 0.5 μ g/ml)
- 11) do same as 5)
- 12) Air dry → mount.

To Page No. 51

Witnessed & Understood by me,

Zoshr

Date

2/21/02

Invented by

J.S

Recorded by

J-S

Date

July/24/01

Page No. _____

Design : changed concentration of treating agent.

① MZ 0.5 μ MNCZ 2.5 μ M

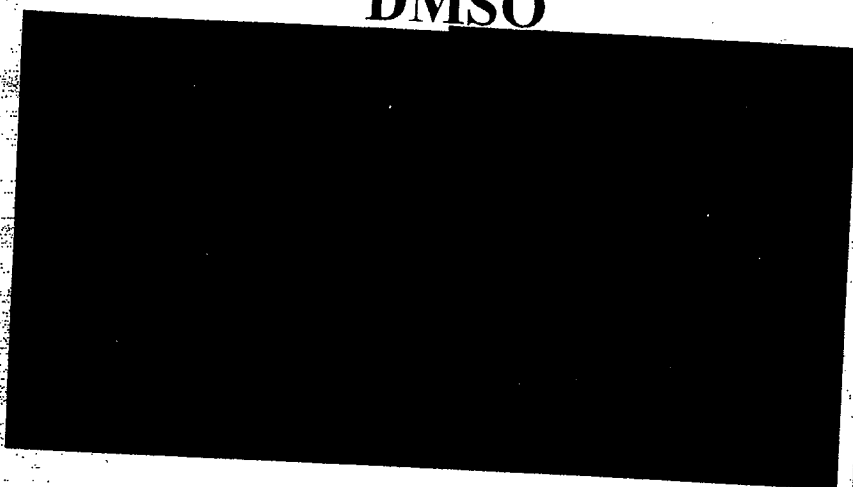
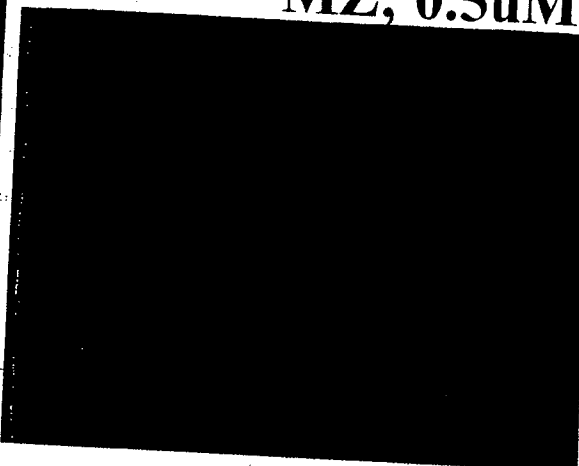
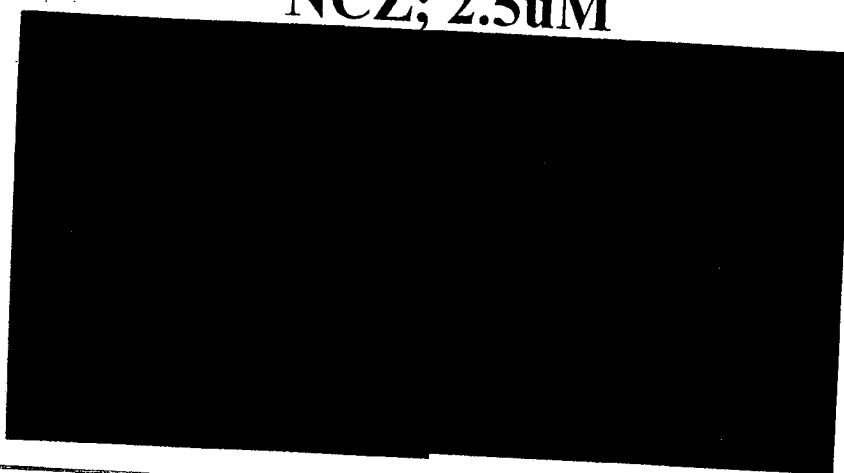
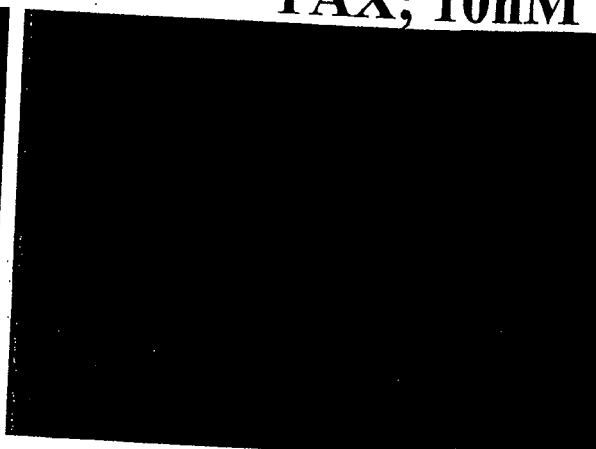
TAX 10 nM

② MZ 5 μ MNCZ 25 μ M

TAX 100 nM

for 3 hours

Tubulin staining in A549 CELL

DMSO**MZ; 0.5 μ M****NCZ; 2.5 μ M****TAX; 10 nM**

& Understood by me,

rphi

Date

2/21/12

Invented by

J-D

Recorded by

J-D

Date

July/26-28

age No. _____

Design

day 0
Seed cells in 12well plate
 5×10^4 / well

day 1
Treatment with
DMSO
MZ 0.1 μ M
NCZ 0.1 μ M
TAX 3 μ M for 3 hours \rightarrow assay.

Lysis buffer with 4 μ g/ml TAX. (7/28)

this time \downarrow It is necessary to reduce of TAX.

H1299 \rightarrow 4 μ g/ml \Rightarrow 2 μ g/ml

H1299 \Rightarrow unstable

H460

A549

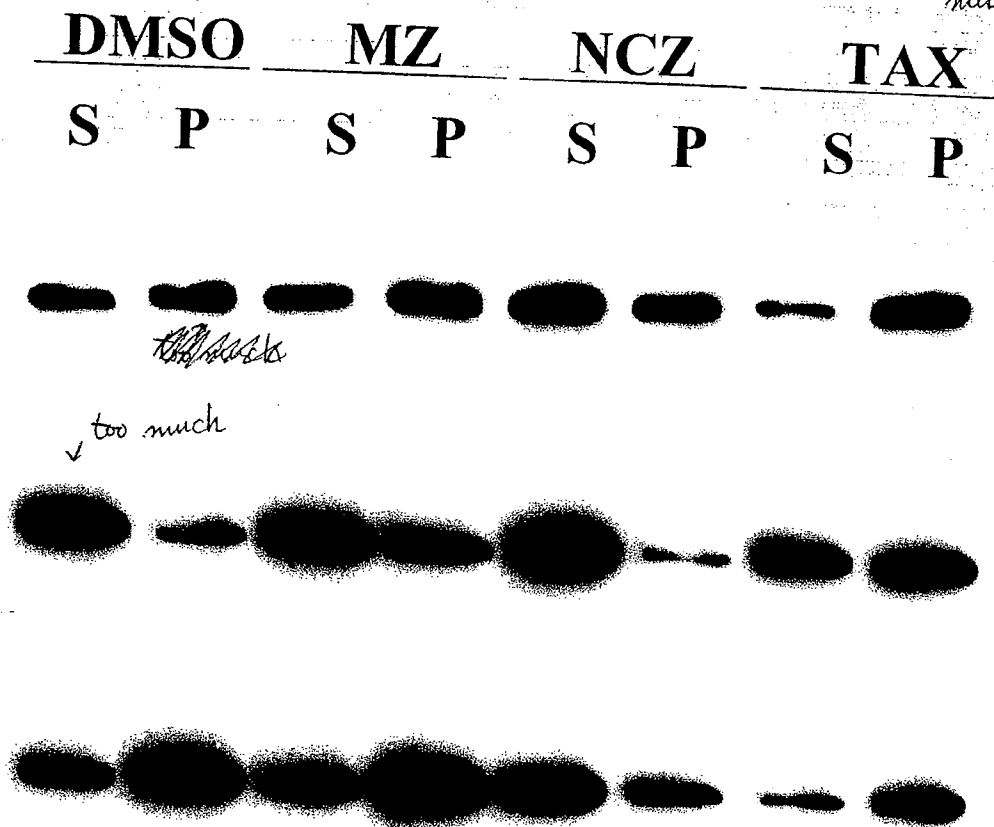
> 4 μ g/ml \Rightarrow 0.5 μ g/ml

2-4 μ g/ml of TAX
must be needed

H460

H1299

A549



ed & Understood by me.

202/hi

Date

2/21/02

Invented by

J.S

Recorded by

J.S

Date

8/1/01

Measurement of soluble and assembled tubulin.

Measurement of soluble (depolymerized) and assembled (polymerized) tubulin was analyzed as described previously with some modifications. Briefly, cells were grown in triplicate wells of a 12-well dish for 24 hours and treated with DMSO or MZ or vinorelbine or paclitaxel for 3 hours. They were then washed with PBS and then lysed with 100 μ l of the lysis buffer (20 mM Tris-HCL, pH 6.8, 0.5% Nonidet P-40, 1 mM $MgCl_2$, 2 mM EGTA, 0.5 μ g/ml paclitaxel).

Following lysis, the cellular residues were scraped from the wells and transferred to a 1.5-ml tube. Each well was rinsed with a second 100 μ l of the lysis buffer and combined with the first. After vigorous vortexing for 10 sec, the samples were centrifuged at 12,000 \times g for 10 min at 4°C. Supernatants containing soluble tubulin were separated from pellets containing polymerized tubulin and placed in separate tubes. The pellets were resuspended in 100 μ l water. The cytosolic and cytoskeletal fractions were each mixed with 200 μ l and 100 μ l of 2 \times SDS sample buffer (125mM Tris-HCL (pH 6.8), 4% SDS, 20% glycerol, 100mM DTT, 2x proteinase inhibitor cocktail (Complete, Roche,)), respectively. Following heating them at 95°C for 5 min, 20 μ l of each sample was analyzed by immunoblotting using both monoclonal mouse anti- α -tubulin and anti- β -actin antibody (Sigma) as described above. The band of each sample was quantiated by NIH image, and the ratios of de-polymerized versus polymerized tubulin were calculated in each treatment sample. The ratio of polymerized versus polymerized actin were also determined as an internal control. The t-test was used to analyze the significant differences between the ratios of depolymerization/polymerization of tubulin or actin in control cells and those in treated cells. Significance was assumed for $p < 0.05$.

2/21/02J-SJ-S8/14/01

From Page No.

- ① To clarify BAX translocation from cytosol to mitochondria
② To estimate microtubule (spindle) formation

Cell = H460 and WI-38.

after MZ treatment

① Method. Using 2x chamber slides.

day 0 → day 1 → day 2

 2×10^4 /well

Treatment

Mito Tracker stain → Immunostaining.

<Mito Tracker staining>

- 1) 100nM in media is prepared using 1mM stock of Mito-red.
- 2) Replace culture media to Mito-containing media
- 3) Incubate for 30min.

↓

Immunostaining protocol (same as P55)

(BAX x 200)

② Method. Same as P55

(<Tubulin x 500>)

Inspected & Understood by me,

Zozhi

Date

2/21/02

Invented by

J-S

Recorded by

J-S

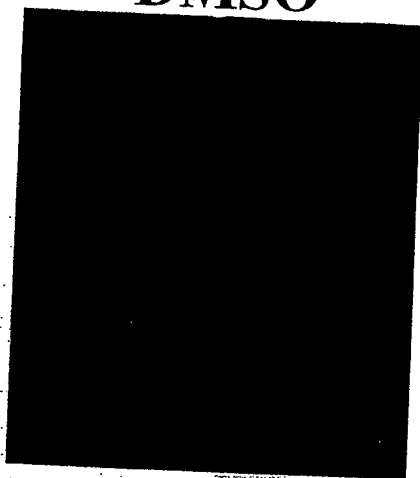
Date

8/5/01

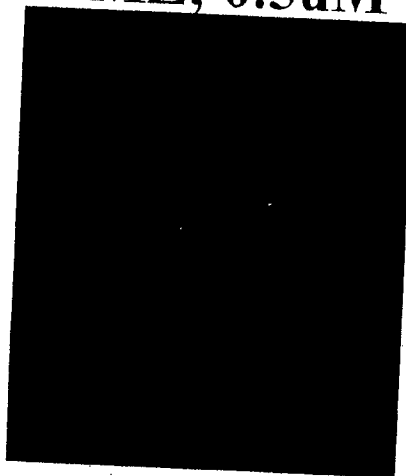
To Page No. 69

Tubulin staining in h460 CELL

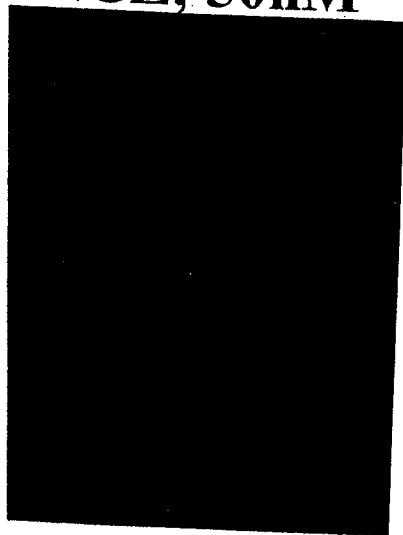
DMSO



MZ; 0.5uM



NCZ; 50nM



TAX; 7nM



W. S. Unpublished, Inc.

Zoshi

Date

2/21/02

Invented by

JS

Recorded by

JS

Date

8/1/01

To Page No. 65

TITLE

In no staining

Project 0.2

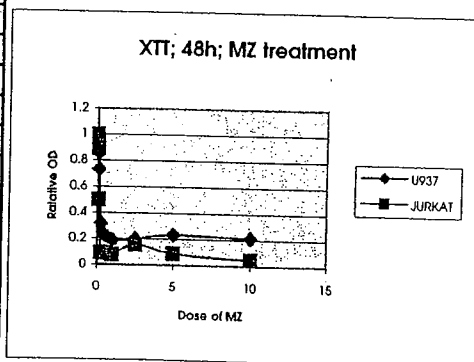
Book No. 2

From Page No. 44

Design : Same as page 41

Cell : JURKAT, U937, MİYAPAKA-2

Row	data	8/7/01 U937	JURKAT					
1	2	3	4	5	6	7	8	
0.13	1.128	1.142	1.067	1.031	0.862	0.449	0.363	C
0.132	1.185	1.026	1.09	0.952	0.842	0.469	0.346	C
0.133	1.073	1.11	1.059	1.003	0.792	0.453	0.379	C
0.135	1.201	1.074	0.986	1.015	0.999	0.438	0.361	C
	0.816	0.783	0.75	0.801	0.477	0.21	0.19	C
	0.873	0.882	0.781	0.769	0.482	0.2	0.251	C
	0.792	0.838	0.875	0.724	0.491	0.209	0.18	C
0.1325	0.947	0.807	0.86	0.81	0.522	0.175	0.166	C
U937	0	0.01	0.025	0.05	0.1	0.25	0.5	C
	0.9955	1.0095	0.9345	0.8985	0.7295	0.3165	0.2305	O.
	1.0525	0.8935	0.9575	0.8195	0.7095	0.3365	0.2135	O.
	0.9405	0.9775	0.9265	0.8705	0.6595	0.3205	0.2465	O.
	1.0685	0.9415	0.8535	0.8825	0.8665	0.3055	0.2285	O.
AVERAG	1.01425	0.9555	0.918	0.86775	0.74125	0.31975	0.22975	C
RATIO	1	0.942	0.905	0.856	0.731	0.315	0.227	C
JURKAT	0	0.01	0.025	0.05	0.1	0.25	0.5	C
	0.6835	0.6505	0.6175	0.6685	0.3445	0.0775	0.0575	O.
	0.7405	0.7495	0.6485	0.6365	0.3495	0.0675	0.1185	O.
	0.6595	0.7055	0.7425	0.5915	0.3585	0.0765	0.0475	O.
	0.8145	0.6745	0.7275	0.6775	0.3895	0.0425	0.0335	O.
AVERAG	0.7245	0.695	0.684	0.6435	0.3605	0.066	0.06425	C
RATIO	1	0.959	0.944	0.888	0.498	0.091	0.089	C
U937	JURKAT							
0	1	1						
0.01	0.942	0.959						
0.025	0.905	0.944						
0.05	0.856	0.888						
0.1	0.731	0.498						
0.25	0.315	0.091						
0.5	0.227	0.089						
1	0.188	0.077						
2.5	0.197	0.155						
5	0.233	0.088						
10	0.211	0.046						



JURKAT	
0	
0.01	C
0.025	C
0.05	C
0.1	C
0.25	C
0.5	C
1	C
2.5	C
5	C
10	C

Invested & Uninvested

To Page No.

yoshi

2/21/02

Invested by

J.S

Date

8/4/01

Invested by

J.S

TITLE Tissue Hemoglobin Assay

Page No. 2

Book No. 2

From Page No. According to users manual
For standard

BECKMAN DU-600

Date: 08/31/01

Time: 01:41

Protein Analysis: Standards

Samples DispStdCurve ViewStats Method SaveClear Print Quit

Assay type: User defined

Standards file: A:\WORK_STD

Component name: ICHIRO

Curve fit: Linear, zero intercept

Sampling device: Auto smplr

Number of standards: 6

Read average time: 0.50 sec

Analytical wl: 600.0 nm

Method name: A:\HEM

Units: MG/DL

Number of replicates: 1

Flag standards over: 1.000% CV

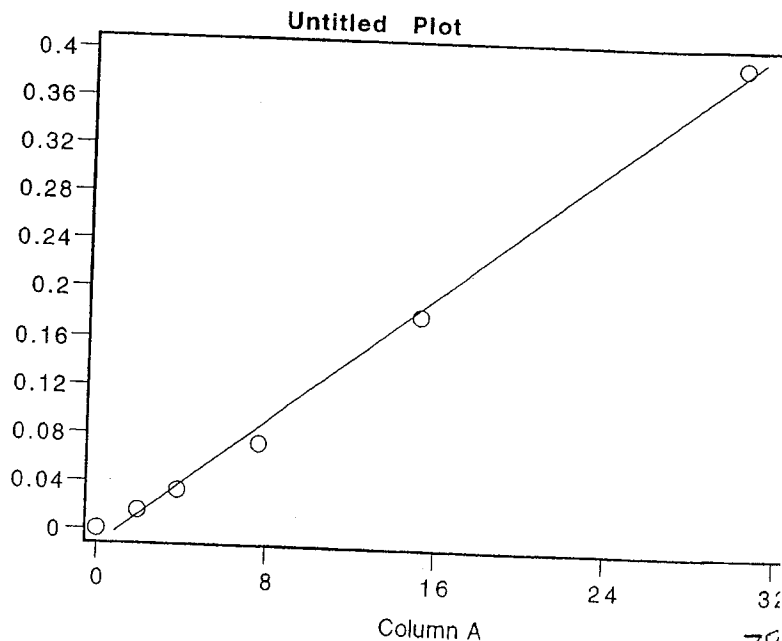
Std#	Rep#	Std Conc	Calc Conc	Diff	%CV	Analytical abs	Use	Flag
Read 1		0.0000	0.1468	0.1468		0.0019	[Y]	
Read 2		1.8750	1.4012	-0.4738		0.0179	[Y]	
Read 3		3.7500	2.7566	-0.9934		0.0352	[Y]	
Read 4		7.5000	5.8401	-1.6599		0.0746	[Y]	
Read 5		15.000	14.2779	-0.7221		0.1823	[Y]	
Read 6		30.000	30.9298	0.9298		0.3948	[Y]	

Tissue Hemoglobin Assay /
Standard

mg/dl	0	1.875	3.75	7.5	15	30
Abs	0.0019	0.0179	0.0352	0.0746	0.1823	0.3948

Column A	Column B
0	0.0019
1.875	0.0179
3.75	0.0352
7.5	0.0746
15	0.1823
30	0.3948

Column B



Inspected & Understood by me,

John

Date

2/21/02

Invented by

J.S

Recorded by

J.S

Date

8/31/01

10 PAGE NO. 78

age No. 27Sample : 5/6/00 Xenograft tumor.

mean average time: 0.00 sec

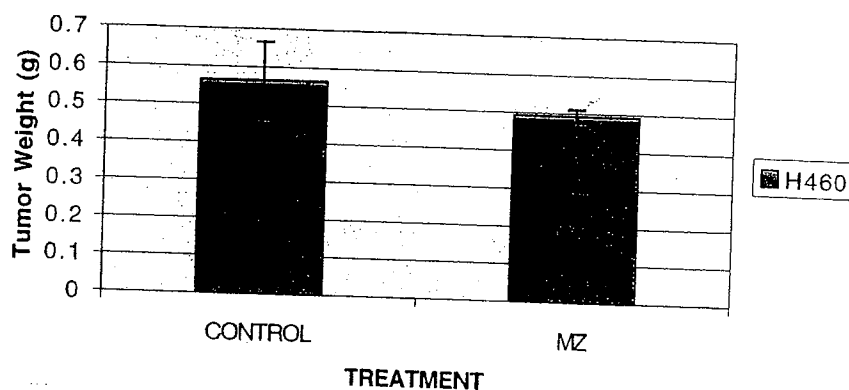
Sample ID	Rept	Analytical abs	H460 5/6/00 Dilution Factor	Conc MG/DL	Flag Tumor volume
1		0.0094			
2		0.0261	C { 1.0000	0.7350	0.42 g
3		0.0223	1.0000	2.0462	0.62 g
4		0.0181	1.0000	1.7479	0.65 g
5		0.0236	M { 1.0000	1.4182	0.47 g
6		0.0078	1.0000	1.8451	0.51 g
			Z { 1.0000	0.6077	0.5 g

TUMOR VOLUME

	CONTROL	MZ
1	0.42	0.47
2	0.62	0.51
3	0.65	0.5
Mean	0.56333333	0.49333333
	0.10208929	0.01699673
		0.39297439

	CONTROL	MZ
	0.56333333	0.49333333

Inhibition of Tumor Growth in vivo

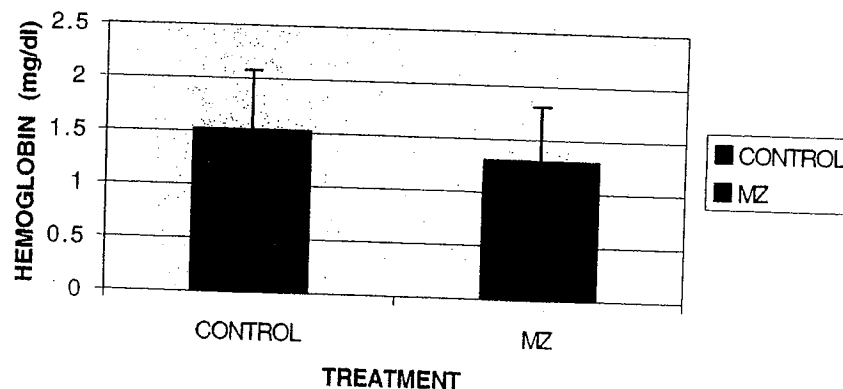


TISSUE HEMOGLOBIN

	CONTROL	MZ
1	0.735	1.4182
2	2.0462	1.8451
3	1.7479	0.6077
Mean	1.5097	1.29033333
	0.56116889	0.51319399
		0.70419558

	CONTROL	MZ
	1.5097	1.29033333

Inhibition of angiogenesis in vivo.



Read & Understood by me,

Zozhi

Date

2/21/02

Invented by

J-S

Recorded by

J-S

Date

8/31/01

To Page No.

From Page No. _____

Re-experiment using same sample as page 78

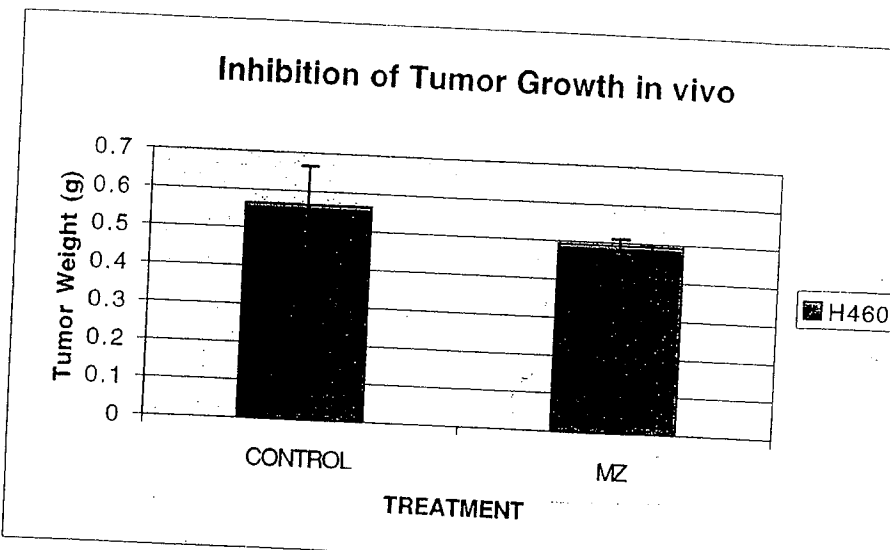
Tissue Hemoglobin Assay 2
 Standard

mg/dl	0	1.875	3.75	7.5	15	30
Abs	0.0004	0.0196	0.0372	0.0773	0.1508	0.3459

TUMOR VOLUME

H460	CONTROL	MZ
1	0.42	0.47
2	0.62	0.51
3	0.65	0.5
average	0.56333333	0.49333333
SE	0.10208929	0.01699673
p		0.39297439

H460	CONTROL	MZ
	0.56333333	0.49333333



TISSUE HEMOGLOBIN

H460	CONTROL	MZ
1	1.75702	2.56919
2	2.30513	1.94098
3	1.80134	1.63065
average	1.95449667	2.04694
SE	0.24859454	0.39041429
p		0.79160352

H460	CONTROL	MZ
	1.95449667	2.04694

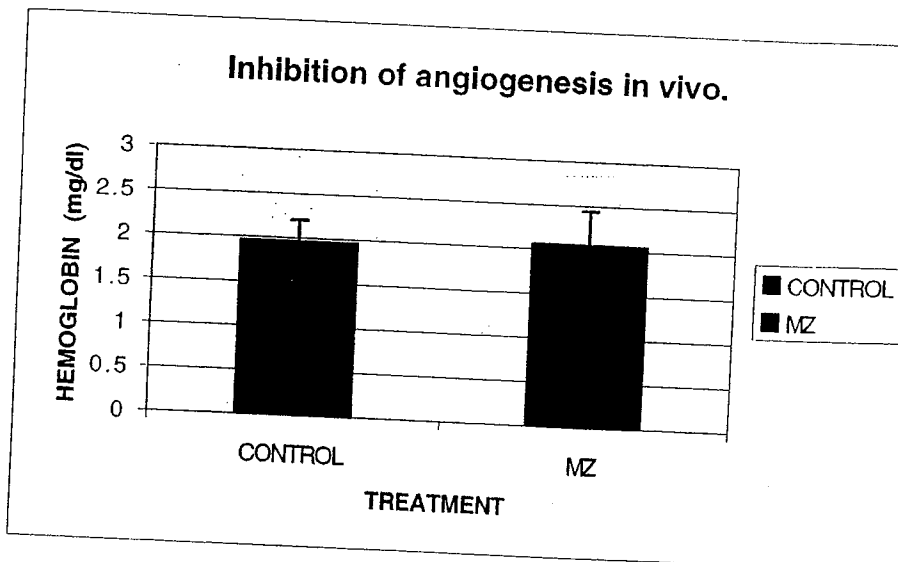


EXHIBIT 3

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TAPAS MUKHOPADHYAY, Ph.D.

Assistant Professor,
M.D. Anderson Cancer Center
1515 Holcombe Blvd., Box 109
(713) 745-4542 - FAX (713) 794-4901
e-mail: tmukhopa@mdanderson.org

October 25, 2001

Dr. Carlo M. Croce
Editor-in-Chief
Cancer Research, AACR
150 S. Independence Mall West
Public Ledger Bldg., Suite 816
Philadelphia, PA 19106-3483

Dear Dr. Croce:

The manuscript entitled "**Mebendazole: A Novel Microtubule Agent Having Potent Antitumor Activity**" is being submitted for consideration for publication as an *Advances in Brief* article in *Cancer Research*. I am sending the cover letter in duplicate, four copies of the manuscript, and four sets of original illustrations. The subject category that applies to this manuscript is Experimental Therapeutics. Please charge in the amount of \$75 for the submission fee in my credit card (VISA 4427-1000-0672-8518, Exp 04/02). I would like to suggest Dr. Jeffrey A. Norton as the Associate Editor.

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♦ Deadline Date: _____

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Mebendazole: A Novel Microtubule Agent Having Potent Antitumor Activity¹

Tapas Mukhopadhyay², Jichiro Sasaki, Rajagopal Ramesh, and Jack A. Roth

Department of Thoracic and Cardiovascular Surgery, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA.

Running Title: A novel microtubule agent as a potent anticancer drug.

Key Words: Tumor growth, angiogenesis, apoptosis, cell cycle, mebendazole

¹Partially supported by grants from the National Cancer Institute, National Institutes of Health (P01 CA78778-01A1, to J. A. R.; a SPORE grant (2P50-CA70970-04); a developmental grant from the National Cancer Institute to The University of Texas M. D. Anderson Cancer Center SPORE grant for lung cancer (P50-CA70907; to T. M.); gifts to the Division of Surgery, at M. D. Anderson Cancer Center from Tenneco and Exxon for its Core Laboratory Facility; M. D. Anderson Support Core Grant (CA 16672); a grant from the Tobacco Settlement Funds as appropriated by the Texas State Legislature (Project 8); the W. M. Keck Foundation; and a sponsored research agreement with Introgen Therapeutics (SR93-004-1).

²Requests for reprints should be addressed at the Department of Thoracic and Cardiovascular Surgery, Box 445, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 745-4542; Fax: (713) 794-4901; E-mail: tmukhopa@mdanderson.org.

²The abbreviations used are: Mebendazole, mebendazole (5-benzoyl-2-benzimidazole carbamic acid). The abbreviations used are: MZ, (methyl 5-benzoylbenzimidazole-2-carbamate; FZ, methyl 5-(phenylthio)-2-benzimidazole carbamate (fenbendazole; PBS, phosphate-buffered saline; NSCLC, non-small cell lung cancer; DMSO, dimethylsulfoxide; SDS, sodium dodecyl sulfate;

4/21/01 - COPY
TO Dr. RAMESH
FOR HT.

ABSTRACT

We have found that Mebendazole (MZ), a derivative of benzimidazole, induces a dose- and time-dependent apoptotic response in human lung cancer cell lines. In this study, MZ arrested cells at the G2/M phase before the onset of apoptosis as detected by using fluorescence-activated cell sorter analysis. MZ treatment also resulted in mitochondrial cytochrome-c release followed by apoptotic cell death. Additionally, MZ appeared to be a potent inhibitor of tumor-cell growth with little toxicity to normal WI38 and human umbilical vein endothelial cells. When administered orally to nu/nu mice, MZ strongly inhibited the growth of human tumor xenografts and significantly reduced the number and size of tumors in an experimental model of lung metastasis. In assessing angiogenesis, we found significantly reduced vessel densities in MZ-treated mice compared with those in control mice. These results suggest that MZ is effective in the treatment of cancer and other angiogenesis-dependent diseases.

INTRODUCTION

Microtubules serve as an intracellular scaffold, and their unique polymerization dynamics are critical for many cellular functions (1; 2). It is conceivable that cytoskeletal dysfunction, manifested as either a disrupted microtubule network or stabilized, "rigid" microtubule cytoskeleton, is an intracellular stress. Furthermore, disruption of the equilibrium between tubulin monomers/dimers and microtubule polymers using microtubule-stabilizing (paclitaxel, docetaxel) or -destabilizing (vinblastine, vincristine, nocodazole, colchicine) agents activates the stress-activated protein kinase signaling cascade. Such microtubule disruption is associated with G2/M-phase blockage (3-7). A number of microtubule drugs have been shown to be highly active, with significant clinical activity against tumor cells. However, the majority of these drugs are highly toxic which limits their application.

We have analyzed both the *in vitro* and *in vivo* effect of mebendazole (5-benzoyl-2-benzimidazole carbamic acid; MZ), a derivative of benzimidazole (BZ), on tumor-cell growth as well as the molecular mechanisms involved in its action. BZ interacts weakly with host tubulin and affects the microtubule assembly only at high concentrations, while MZ is an anthelmintic drug that is used extensively for gastrointestinal parasitic infections in humans. However, the major application of these compounds to date, has been the treatment of veterinary and human helminthiasis, in which they have demonstrated remarkable efficacy and safety (8). In this study though, we were interested in determining the effect of MZ on solid tumor growth and angiogenesis. Structurally different from other anticancer drugs, MZ is remarkably safe at high doses in humans. We report here evidence indicating that MZ induces G2/M cell cycle arrest, which ultimately promotes apoptosis in lung cancer cells. Our results demonstrate for the first time the antitumor and antiangiogenic effects of MZ both *in vitro* and *in vivo*.

MATERIALS AND METHODS

***In Vitro* Cell Culture and Proliferation Assay.** Cells of the human non-small cell lung cancer cell line A549 and WI38 normal fibroblasts (American Type Culture Collection, Rockville, MD) and H460 cells (a gift from Drs. Adi Gazdar and John Minna, The University of Texas Southwestern Medical Center at Dallas, Dallas, TX) were seeded on to culture plates (2 x 10⁴ cells/well) in F12 and RPMI medium, respectively, supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Also, human umbilical vein endothelial cells (HUVEC) were grown in medium supplemented with growth factor (Clonetics, San Diego, CA). When grown to 40% - 50% confluence, the cells were exposed to MZ dissolved in dimethyl sulfoxide (DMSO). Cell growth was monitored by counting the viable cells using a hemocytometer.

DNA Fragmentation and Cell Cycle Analysis. Control and MZ-treated cells were washed in cold phosphate-buffered saline (PBS). The cell pellets were lysed in lysis buffer (10 mM Tris, pH 7.4, 10 mM ethylenediaminetetra acetic acid, pH 8.0, 0.5% Triton X-100) and incubated for 10 min at 4°C and then incubated with 200 µg/ml RNase A for 1 h at 37°C. Following centrifugation, the supernatants were incubated with 200 µg/ml Proteinase K for 30 min at 50°C. Next, DNA fragments were precipitated with 0.5 M NaCl and 50% isopropanol and the samples were loaded in 2% agarose TBE gel and stained with ethidium bromide.

Growth of Tumor Xenografts in Nude Mice. Prior to the start of the experiments, mice underwent total-body irradiation (3.5 Gy). One day later, all animals received an injection of 2 x 10⁶ H460 tumor cells into the lower right flank. Starting on the day after a 3- to 5-mm tumor was established, we administered an oral suspension of MZ at the indicated concentration every other day; five mice were used in each group. Both control and treated mice were then

monitored for tumor growth, with cross-sectional diameter of the tumors measured externally every 7 days. Also, the tumor volume was calculated as described previously (9). The experiments were conducted in triplicate.

Hemoglobin Assay. Quantitation of tumor vascularity was performed by using hemoglobin assay essentially as described previously (10). Briefly, subcutaneous tumors were excised, weighed, individually frozen in test tubes, and usually 24 h later, thawed. Approximately 20 ml of distilled water/gram of tumor tissue was then added, and the tumor was homogenized using a blade homogenizer until it was fully disintegrated. The debris was then pelleted via centrifugation (3000 x g, for 5 min), and the supernatant, which contained hemoglobin, was collected. The concentration of hemoglobin in the supernatant was determined according to the catalytic action of hemoglobin on the oxidation of 3,3',5,5'-tetramethylbenzidine by hydrogen peroxide as outlined by the manufacturer (Plasma Hemoglobin Kit; Sigma Chemical Co. St. Louis, MO)

Evaluation of Lung Metastases and Treatment *In Vivo*. To establish lung metastases, A549 tumor cells (10^6) were injected into the tail vein of 10 female nude mice as described previously (11). Six days later, we divided the mice into two groups of five each. Group 1 received no treatment, while group 2 received 1 mg of MZ orally (100 μ l) twice a week for 3 weeks. After the 3 weeks, the animals were killed via CO₂ inhalation. The mice's lungs were then injected intratracheally with India ink and fixed in Fekete's solution. The therapeutic effect of MZ treatment was determined by counting (without knowledge of which was the treatment group) the metastatic tumors in each lung under a dissecting microscope. The experiments were performed three times, and data were analyzed and interpreted as being statistically significant if the *P* value was < 0.05 according to the Mann-Whitney test.

Histologic Analysis of Blood Vessels in Tumor Xenografts. Five-micrometer sections of paraffin-embedded tissue samples were stained with hematoxylin and eosin and subjected to immunoperoxidase detection of endothelial cells using a CD31 antibody (12). Vascular areas that stained positively for CD31 (at least five fields per specimen) were analyzed under bright-field microscopy. In all of the staining procedures, we included appropriate negative controls.

Chamber Assay of Angiogenesis. We used the dorsal air-sac method (13) to assay angiogenesis *in vivo*. Briefly, 1×10^7 cultured A549 cells were suspended in PBS and packed into round cellulose-ester membrane chambers having a diameter of 14 mm (pore size, $0.45 \mu\text{m}$; Millipore Corporation, Bedford, MA). Each chamber was then implanted into a dorsal air sac of a nude mouse. From the next day onward, after implantation, the mice were given an oral suspension of MZ (1 mg/mouse/day); five mice were used in each group. The mice were killed on day 5, and the subcutaneous region overlying the chamber in each mouse was photographed.

Cellular Fractionation for Cytochrome-c and Western Blot Analysis. We performed cell fractionation using the Apo Alert Cell Fractionation Kit (Clontech, Palo Alto, CA) according to the user's manual. In brief, tumor cells were harvested and washed in washing buffer and then homogenized in lysis buffer in an ice-cold Dounce tissue grinder. The cell homogenates were then centrifuged at $700 \times g$ for 10 min at 4°C . Afterwards, the supernatants were transferred into 1.5-ml tubes and centrifuged at $10,000 \times g$ for 25 min at 4°C ; they were then collected as cytosolic fractions, and the pellets were lysed in lysis buffer and collected as mitochondrial fractions. Western blot analysis was done as described earlier (14). Briefly, the protein concentration in both fractions was determined using the Bradford method (Bio-Rad, Hercules, CA). Next, $25 \mu\text{g}$ of protein was fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to Amersham

membranes (Amersham, Arlington Heights, IL), and immunoblotted with monoclonal antibodies against cytochrome-*c*, cytochrome-*c* oxidase subunit IV (COX IV), and β -actin. Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham).

Statistical Analysis. To summarize the study results, we reported descriptive statistics, such as the mean and standard deviation. Also, two-sample *t*-test was performed to compare the tumors in control mice with those in mice treated under various conditions.

RESULTS

Effect of MZ on Tumor Cell Growth *In Vitro*. MZ treatment strongly inhibited the growth of the lung cancer cell lines (Fig. 1A); the half-maximal inhibitory concentration (IC_{50}) was $\sim 0.16 \mu M$. Specifically, MZ induced dose- and time-dependent inhibition of the growth of these cells. However, although MZ was highly cytotoxic to the tumor cells in culture, reducing their number to below the initial plating density, it had no effect on normal HUVEC or WI38 fibroblasts even at a concentration of $1 \mu M$ (Fig. 1A). Additionally, we examined the effect of MZ on H460 and A549 human lung cancer cells in a 5-day growth assay (Fig. 1B). We found that MZ inhibited growth of the cells fivefold compared with that of control cells. The growth-inhibitory effect was not restricted to lung cancer cells, as MZ also profoundly inhibited growth of breast, ovary, and colon carcinomas and osteosarcomas (Table 1), producing IC_{50} values that varied from 0.1 to $0.8 \mu M$ (data not shown). Table 1 also shows the growth-inhibitory effects of MZ on a number of tumor cell lines after 48 h of treatment.

Induction of G2/M Arrest Followed by Apoptosis by MZ in Lung Cancer Cell Lines. When cells were treated with varying doses of MZ, they were killed due to apoptosis. Specifically, H460 cells were exposed to MZ (0.2 to $5.0 \mu M$) for 24 h before the DNA was

extracted for agarose gel electrophoresis. Figure 1C shows that MZ induced DNA fragmentation at 24 h in a dose-dependent manner. The mechanism of cell death was determined to be apoptosis via detection of apoptotic cell populations that displayed a sub-2N genomic content during fluorescence-activated cells sorting (Fig. 1D). We found that the cells were blocked at the G2/M phase 12 h after MZ treatment before undergoing apoptosis. Cells were rounded and partly detached after 12 h of MZ treatment. However, after 24 h of treatment, cell shrinkage occurred, and nuclear bodies were evident; subsequently the cells underwent apoptosis. After 48 h of MZ treatment, more than 60% of the cells had undergone apoptosis with characteristic nuclear fragmentation (Fig. 1D). In addition, a number of apoptotic gene family proteins were examined using western blot analysis. It was found that p53 protein is post-translationally stabilized and elevated without an increase in mRNA (data not shown). This occurs so frequently during apoptosis induced by diverse stress stimuli that it has been considered a common feature of the apoptotic process. Furthermore, as a consequence of p53 stabilization, expression of the p53 target genes p21 and MDM2 was also induced. However, MZ had no effect on genes belonging to the Bcl2 family, including Bcl-xl, bax, bad, and bak, as determined via western blot analysis (data not shown).

Induction of Cytochrome-c Release and Caspase Activation by MZ. Because MZ could inhibit the growth of p53-null cell lines and other p53-mutated cells, although at a higher dose, we examined the other p53-independent pathways. To examine whether MZ signaling goes through a mitochondrial pathway, H1299 (p53-null) and H460 (wild-type) cells were treated with MZ in a dose-dependent manner, and cytosolic extracts lacking mitochondria were prepared and analyzed via immunoblotting (Fig. 1E). Cytochrome-c accumulated in cytosolic extracts at 12 h after exposure to MZ increased in both of the cell lines in a dose-dependent

manner. Also, the membranes were probed using an antibody against Cox IV, a protein that is specific for mitochondria, as an internal control. Both cell lines showed an increase in cytochrome-c protein in the cytoplasm after MZ treatment in a dose-dependent manner. Twenty-four hours after MZ treatment, activation of caspase-9 and caspase-8 and cleavage of the caspase substrate PARP and procaspase 3 were detectable (data not shown).

Inhibition of Tumor Cell Growth and Angiogenesis by MZ. The effect of MZ on the proliferation of tumor-cell lines *in vitro* prompted us to investigate its antitumor activity in a nu/nu mouse model. We established tumors in the mice by subcutaneously injecting them with 1×10^6 H460 cells, which are human non-small cell lung cancer cells. A dose-escalation study indicated that MZ suppressed growth of the tumors in a dose-dependent manner (Fig. 2A). Specifically, mice having established tumors (~3 mm in diameter) were fed 1 mg of MZ orally every other day, which was sufficient to profoundly inhibit tumor growth (Fig. 2B). The tumors were then harvested, photographed (Fig. 2C), and weighed. The experiment was repeated two times using 10 animals in both the control and treatment group. We found a marked difference in tumor weight between the MZ-treated and control animals (Fig. 2D). Additionally, in control mice, the xenograft of H460 cells exhibited a marked increase in tumor-growth kinetics compared with that in mice in the MZ-treated group. Furthermore, MZ-treated mice showed no signs of toxicity and were all healthier than the control mice were, during the 4 weeks of treatment (data not shown).

To determine whether the differences in growth kinetics observed *in vivo* were associated with variations in tumor vascularity, sections of subcutaneous tumors established from H460 cells were stained for CD31, a marker expressed by endothelial cells. A significant decrease in the number of CD31-positive endothelial cells was observed in MZ-treated mice when compared

with control mice. This analysis demonstrated substantially increased blood-vessel density in untreated mice compared with that in MZ-treated mice (Fig. 2E). Thus, MZ treatment profoundly reduced the neovascularization and growth of human lung cancer xenografts in nude mice. This tumor-suppressing effect of MZ may have been due to inhibition of tumor-induced angiogenesis. In addition, the tumor vascularity *in vivo* was quantitated in control and MZ-treated mice using a hemoglobin assay. The results of this assay indicated that there was a 75% reduction in hemoglobin content per gram of tumor sample obtained from MZ-treated mice, as compared with control mice (Fig. 2F).

Angiogenesis *in vivo* was further assayed using the dorsal air-sac method (15) by photographing the area of subcutaneous neovascularization in mice overlying a semipermeable membrane chamber containing H460 or A549 cells. Twenty-four hours after each chamber was implanted, the animals were fed 1 mg of MZ orally every other day for a total of three treatments. Both the number and caliber of the blood vessels were significantly reduced in mice treated with MZ compared with those in control mice (Fig. 2G). To exclude the possibility that the reduced vasculature was due to a lack of viable tumor cells in the chamber, we prelabeled tumor cells using a fluorescent dye before injecting them into the chamber. After photographing the blood vessels, we examined the tumor cells on the membrane using a fluorescent microscope. The results indicated that the control and MZ-treated mice had similar cell densities on the membrane filters (Fig. 2H).

Next, we sought to determine whether MZ treatment would inhibit the growth of human lung cancer colony formation in an experimental lung metastasis model. In this study, about 300 metastatic colonies appeared in the lungs of control mice, 21 days after the injection of A549 cells via the tail vein (Figs. 3A and 3B). However, the oral administration of 1 mg of MZ per

mouse every other day reduced the mean colony count to 80% of the mean count in control mice ($P < 0.0001$). This experiment was performed three times with similar results. In another experiment, mice that were treated using paclitaxel alone did not show a significant reduction in colony formation (data not shown). Histochemical staining of lung tissues using hematoxylin and eosin indicated that not only the number but also the size of the metastatic tumor colonies (as measured according to the transverse diameter of the tumor colony) was substantially reduced by treatment using MZ (Fig. 3C).

Discussion

MZ is one of the truly broad-spectrum anthelmintics, the BZs, which have a high therapeutic index. Central to the success of the BZs is their selective toxicity in helminths. Although the diverse activities of these compounds have been described at both the biochemical and cellular level, their molecular mechanism of action has not been explored in detail; when it has been studied, this mechanism has proven to be controversial. BZs are known to inhibit a wide variety of apparently unrelated mechanisms. Of these mechanisms, fumarate reductase, glucose uptake, and microtubule inhibition satisfy many of the criteria considered relevant for a putative site of action. This gives rise to the question of whether these mechanisms are directly or indirectly related. Based on the inhibitor profile of both fumarate reductase and glucose uptake, it is apparent that these systems are not specific to BZs. The coincidence of three structurally distinct microtubule-inhibitor classes acting on these mechanisms supports the hypothesis of microtubule dependence; however, this is complicated by the activity of noninhibitors in both techniques.

There are sufficient data supporting a general concept of primary microtubule action leading to a series of biochemical effects that either directly or indirectly elicit a number of

changes; as we demonstrate here, these changes vary in normal and cancer cells. The results of binding studies using enriched extracts from the tubulin of helminths and mammals have suggested that tubulin are the substrate of BZs (8; 16; 17). However, the results of crystallographic and other studies have indicated that the tubulin-binding site of BZs is distinctly different from that of other microtubule-disrupting agents like vinblastine and paclitaxel (taxol). Drugs in the latter group bind to tubulin at sites located near the intradimer interface and facing the lumen of the microtubule, whereas the possible binding site for BZs is on the outside of the microtubule (18; 19). Although BZs are potent inhibitors of tubulin, a comparison of their relative activity showed that some structural refinement produces BZ derivatives, like MZ, that are significantly less active against mammalian tubulin, although their broad structural specificity remains unchanged. Also, some BZs have been reported to have poor systemic absorption after oral administration *in vivo*; the exception is MZ, which has been shown to have an absorption profile of >50% (20). The observed safety of the BZs as anthelmintics may also be unrelated to BZ-tubulin binding but rather may be due to differences in the metabolic or detoxification pathways as suggested by (21). For example, rapid, extensive metabolism of BZs into less-toxic metabolites (*e.g.* sulfoxides and sulfones) by the hepatic microsomal enzymes (22; 23) may account for some of the lack of host toxicity. Parasites, on the other hand, lack these metabolic pathways and are killed by BZs. Additionally, some terbenzimidazole compounds have been reported to be topoisomerase I poisons (24); therefore, we tested MZ for such an effect but could not detect it (unpublished results).

The effect of MZ as an antitumor agent has never been tested before. With that in mind, one of our most encouraging findings was that MZ inhibited neovascularization both *in vitro* and in the human xenografts we tested, indicating that MZ is a potent antiangiogenic agent.

Moreover, it had no effect on normal endothelial cell growth but directly targeted tumor cells *in vivo*. Although the molecular mechanism of MZ action on tumor-growth inhibition requires further elucidation, our results show that MZ may be effective in the treatment of cancer and other angiogenesis-dependent diseases.

Acknowledgments

We thank Marjorie Johnson for her technical assistance and Carmelita Concepcion and Peggy James for preparation of the manuscript.

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Table 1. *Growth-inhibitory effect of MZ on other human tumor cell lines^a*

Cell line	Tumor origin	Cell survival (%)
MCF-7	Breast	24.26 ± 8.4
H322	Lung	68.50 ± 3.7
H226Br	Lung	64.24 ± 0.2
H358	Lung	73.45 ± 1.8
Saos-2	Osteosarcoma	65.36 ± 10.1
SW480	Colon	63.63 ± 13.3
MDA 231	Breast	66.24 ± 15.8
SK-OV-433	Ovary	28.15 ± 6.1
RD	Rhabdomyosarcoma	72.24 ± 2.8
HT1080	Osteosarcoma	44.49 ± 7.7

^aThe viability of the cells was measured using a trypan blue exclusion assay after 48 h of MZ treatment (0.16 μ M). The 100% value was derived from measurements obtained from untreated cells. Experiments were done in triplicate.

FIGURE LEGENDS

Fig. 1. Effect of mebendazole (MZ) on cell growth and apoptosis. **(A)**, Dose-dependent inhibition of cell proliferation after MZ treatment. The H460, A549, HUVEC, and WI38 cell lines were used in this assay. **(B)**, H460 and A549 cells were treated with 0.165 μ M MZ (IC_{50}), and a 5-day growth assay was done. **(C)**, dose-dependent DNA fragmentation analysis was done in H460 cells after 24 h of MZ treatment. Lane 1, controls; lanes 2-4, H460 cells exposed to 0.2, 0.5, and 1.0 μ M MZ. **(D)**, H460 cells treated with 0.2 μ M MZ, harvested at different time intervals, and stained with PI. The cells were processed for fluorescence-activated cell sorter analysis to determine the cell cycle phase and apoptosis. Subdeplod populations indicated the apoptotic cells. The phase-contrast photomicrographs show mitotic cells after 12 h of MZ treatment and apoptotic nuclei after 24 and 48 h of MZ treatment. **(E)**, Cytochrome-*c* detected using western blot analysis in the cytosolic fraction of H1299 and H460 cells. A considerable increase in cytochrome-*c* was noticed, which correlated with the MZ dose. An antibody against COX IV, a mitochondria specific protein, was used to probe the membrane to eliminate the possibility of contamination during fractionation.

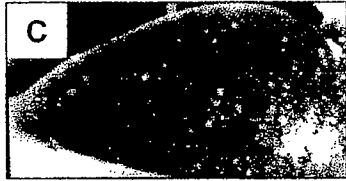
Fig. 2. Effect of MZ on tumor growth and angiogenesis. **(A)**, MZ inhibited H460 xenograft tumor growth in athymic nu/nu mice in a dose-dependent manner. H460 cells were injected into mice (2×10^6 cells/mouse), and mice having established tumors (3-4 mm in diameter) were fed different concentrations of MZ (T02, T04, and T08 represent 200, 400, and 800 μ g of MZ, respectively) every other day while control animals received

PBS. **(B)**, Significant growth inhibition was observed when mice were fed 1 MS of MZ every other day. Open circle control mice; closed circle, MZ-treated mice. **(C)**, Tumors were excised from control and MZ-treated mice after 4 weeks and photographed. A few mice were killed at the start of treatment when the tumor had reached 3-4 mm in diameter **(D)**, Graphic representation of the weight ($\text{mg} \pm \text{standard deviation}$) of tumors in control and MZ-treated mice on day 28. **(E)**, Histologic analysis of blood vessels in H460 xenograft tumors via immunoperoxidase detection of endothelial cells using a CD31 antibody. Con. control mouse; MZ-treated mouse. **(F)**, Plot of milligrams of hemoglobin per gram of tumor tissue obtained from control and treated animals following hemoglobin assay. **(G)**, Effect of MZ on angiogenesis *in vivo*. Chamber assay showing that MZ inhibited capillary formation using A549 cells. It should be noted that the control implant had a tree-like architecture of major vessels (arrows) connecting to minor branches (arrowheads) but that the MZ-treated implants had scarce vessels. Con, control; MZ, treated. **(H)**, A549 cells prelabeled using a fluorescent cell marker were detected on the chamber membranes of control (Con) and MZ-treated (MZ) mice.

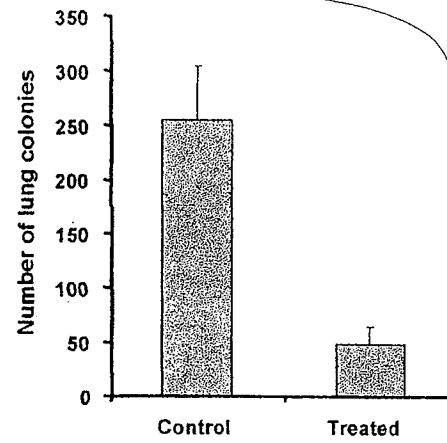
Fig. 3. Effect of MZ treatment on lung colony formation in an experimental metastasis model.

(A), A549 cells formed colonies on lung surfaces when injected through the tail vein. C, control mouse; T, MZ-treated mouse. The white spots on the lung surfaces are colonies (arrows). **(B)**, Quantitation of lung colonies in control and MZ-treated animals ($P < 0.0001$). **(C)**, Hematoxylin and eosin-stained lung sections showing the sizes of tumor colonies in control (Con) and MZ-treated (MZ) animals. The colonies are indicated by dotted lines.

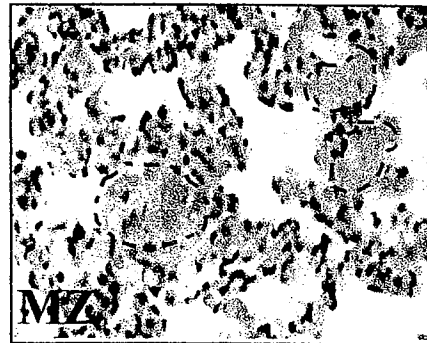
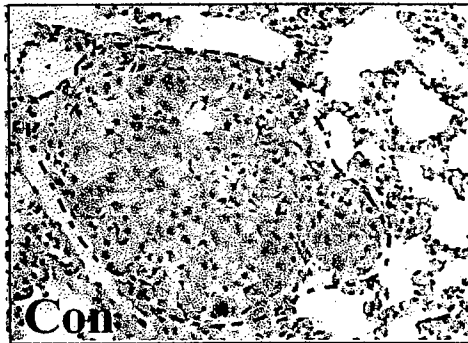
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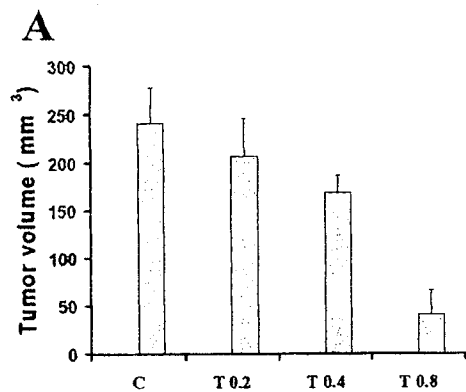


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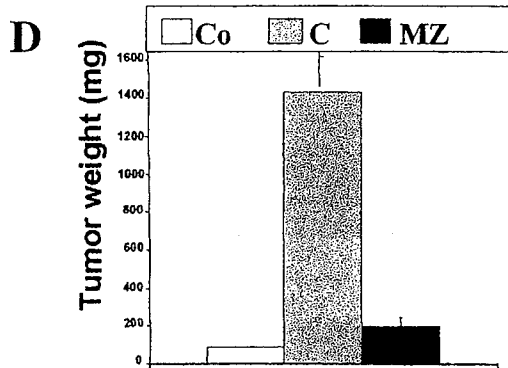
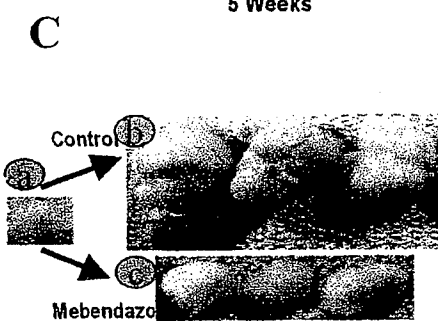
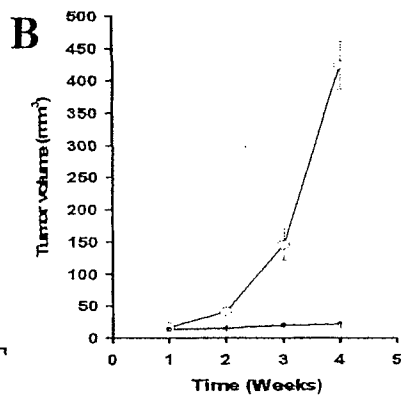


C





5 Weeks



H460 tumor

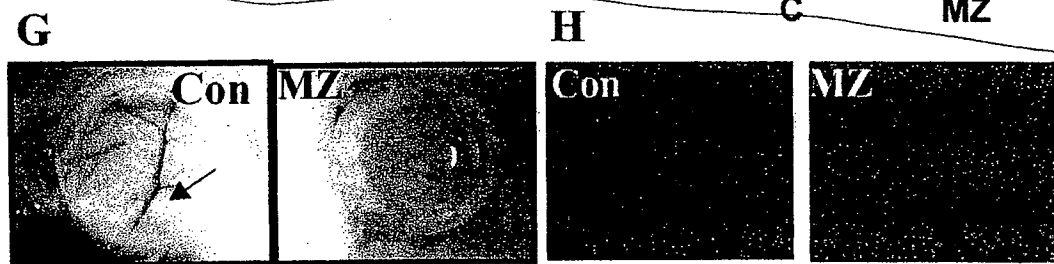
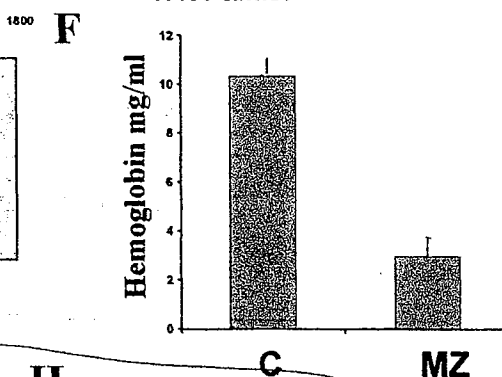
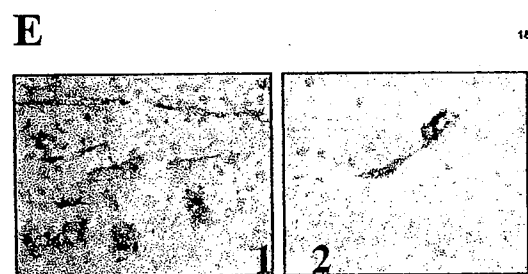


EXHIBIT 6



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/043,877	01/09/2002	Tapas Mukhopadhyay	INRP:095US 10200175	6285

7590 07/10/2006
FULBRIGHT & JAWORSKI L.L.P.
SUITE 2400
600 CONGRESS AVENUE
AUSTIN, TX 78701

EXAMINER

FETTEROLF, BRANDON J

ART UNIT	PAPER NUMBER
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1642

DATE MAILED: 07/10/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

FULBRIGHT & JAWORSKI, LLP

IPT DOCKETING

Docketed ☒ Not Req'd ☐ Confirmation ☐

Initials 1st: *fm* Initials 2nd: *g*

JUL 12 2006

Attorney *SLH, MAD*

Docket No. *INRP:09505*

Action Req'd Date Due

*3mo OA initial
deadline 10/10/06
final 1/10/07*

Office Action Summary	Application No. 10/043,877	Applicant(s) MUKHOPADHYAY ET AL.	
	Examiner Brandon J. Fetterolf, PhD	Art Unit 1642	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 28 April 2006.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 2-8, 10, 11, 13-63, 65-74, 76-160, 163, 166, 168 and 171-175 is/are pending in the application.
- 4a) Of the above claim(s) See Continuation Sheet is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 2, 3, 10, 13-19, 21-29, 76, 77, 83-97 and 99-106 is/are rejected.
- 7) ☒ Claim(s) 20 and 98 is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. _____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- | | |
|--|---|
| 1) <input checked="" type="checkbox"/> Notice of References Cited (PTO-892) | 4) <input type="checkbox"/> Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____ |
| 2) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948) | 5) <input type="checkbox"/> Notice of Informal Patent Application (PTO-152) |
| 3) <input type="checkbox"/> Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08)
Paper No(s)/Mail Date _____ | 6) <input type="checkbox"/> Other: _____ |

Continuation of Disposition of Claims: Claims withdrawn from consideration are 4-8,11,30-63,65-74,78-82,107-160,163,166,168 and 171-175.

Response to the Amendment

The Amendment filed on 4/28/2006 in response to the previous Non-Final Office Action (11/16/2005) is acknowledged and has been entered.

Claims 2-8, 10-11, 13-63, 65-74, 76-160, 163, 166, 168, 171-175 are currently pending.

Claims 4-8, 11, 30-63, 65-74, 78-82, 107-160, 163, 166, 168 and 171-175 are withdrawn from consideration as being drawn to a non-elected invention and/or species.

Claims 2-3, 10, 13-29, 76-77 and 83-106 are currently under consideration.

The Declaration filed on 4/28/2006 under 37 CFR 1.131 has been considered but is ineffective to overcome the Camden (US Patent No. 6,262,093) reference.

The Camden reference is a U.S. patent or U.S. patent application publication of a pending or patented application that claims the rejected invention or an obvious variant. An affidavit or declaration is inappropriate under 37 CFR 1.131(a) when the reference is claiming the same patentable invention or are obvious variants, see MPEP § 608 and 2306. If the reference and this application are not commonly owned, the reference can only be overcome by establishing priority of invention through interference proceedings. See MPEP Chapter 2300 for information on initiating interference proceedings. If the reference and this application are commonly owned, the reference may be disqualified as prior art by an affidavit or declaration under 37 CFR 1.130. See MPEP § 718.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office Action.

Rejections Maintained:

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

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Claims 76, 83-97 and 99-106 **remain** rejected under 35 U.S.C. 103(a) as being unpatentable over Camden (US 6,262,093, 1999) in view of Perdoma *et al.* (J. Cancer Res. Clin. Oncol. 1998, 124, 10-18).

Camden teaches (column 11, line 69 to column 12, line 51) a method of inducing apoptosis in cancer cells expressing abnormal p53 by administering an effective amount of a benzimidazole derivative. The patent further teaches (column 12, line 52 to column 13, line 24) a method of treating a patient having cancer expressing abnormal p53 by administering an effective amount of a benzimidazole derivative to induce apoptosis. Moreover, Camden discloses (column 14, line 53 to column 24, line 31) a method of treating a patient with cancer comprising administering an effective amount of a benzimidazole derivative. With regards to the cancer, the patent teaches that cancer includes, but is not limited to, cancers of the breast, lung, non-small cell lung and sarcoma (column 3, lines 45-50) or cancer that has survived treatment with another anticancer agent (column 29, lines 9-13). Specifically, Camden discloses the apoptotic effect in cancer cells such as, for example, MCF7 breast cells both in vitro (column 12, lines 46-51) and in vivo (column 16, lines 48+). With regards to the cancer cells, the patent teaches (column 12, lines 46-51) that some of the cancer cell lines tested are known to express abnormal p53. With regards to administration, Camden provides that 1 to 1000 mg/kg of a benzimidazole derivative (column 5, line 58 to column 6, line 17) can be administered orally, by intravenous injection, by parental administration or by injection into or around the tumor (column 6, lines 26-43). In addition, Camden teaches that the compound can be administered as a single daily dose or repeated at least once (column 6, lines 18-25). Furthermore, the patent shows that even at a concentration less than 10 µg/mL, the benzimidazole derivatives were capable of inducing apoptosis in p53 abnormal cell lines (column 12, lines 46-51). Thus, it does not appear that the claim language or limitation results in a manipulative difference in the method steps when compared to the prior art disclosure. See Bristol-Myers Squibb Company v. Ben Venue Laboratories 58 USPQ2d 1508 (CAFC 2001).

Camden does not teach determining the tumor suppressor status by way of Southern blotting, Northern blotting, PCR, ELISA or Western blotting (claims 23-28 and 101-106).

Perdoma *et al.* teach determining the p53 status, by Western blot analysis (page 12, 3rd paragraph) or other methods such as polymerase chain reaction (PCR), could make it possible to predict the response to therapy in certain patients (page 17, 1st column, 2nd paragraph). Perdoma *et*

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al. further teach that the response to cisplatin *in vivo* of NSCLC tumor lines was dependent on p53 status (page 17, 1st column, 2nd paragraph). Specifically, the reference teaches wt-p53 tumors showed a regression in size of around 60%, whereas mt-p53 tumors stopped growing (page 17, 1st column, 2nd paragraph).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to determine the status of a tumor suppressor gene, like p53, in a tumor cell prior to administering a benzimidazole derivative using techniques such as Western blot, PCR or other methods of analysis. One would have been motivated to do so because Camden teaches the selectivity in killing p53 abnormal cell lines versus cells expressing normal p53 (column 12, lines 52+), while Perdoma *et al.* teaches that the “response to cisplatin *in vivo* of tumors derived from different NSCLC lines was dependent on p53 status (page 17, 1st column, 2nd paragraph).” Further, one of ordinary skill in the art would have a reasonable expectation of success because Perdoma *et al.* teaches “analysis of p53 status, by immunohistochemical or other methods such as the polymerase chain reaction (PCR), could make it possible to predict the response to therapy in certain patients (page 17, 1st column, 2nd paragraph).”

In response to this rejection, Applicants contend that Camden is not prior art because Applicants have demonstrated reduction to practice of the claimed invention before the priority date of Camden I, conceived of their invention before the priority date of Camden, and were diligent to the time of the priority date of the instant application as evidenced by the three declarations under 37 CFR 1.131. As such, the rejection of claims 76, 83-97 and 99-106 under 35 USC 103 cannot be sustained.

These arguments have been carefully considered, but are not found persuasive because, as noted above, an affidavit or declaration is inappropriate under 37 CFR 1.131(a) when the reference is claiming the same patentable invention, see MPEP § 2306. As such, claims 76, 83-97 and 99-106 **remain** rejected under 35 U.S.C. 103(a) as being unpatentable over Camden (US 6,262,093, 1999) in view of Perdoma *et al.* (J. Cancer Res. Clin. Oncol. 1998, 124, 10-18).

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New Rejections Necessitated by Amendment:

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 76-77, 83-97, 99-106 are rejected under 35 U.S.C. 103(a) as being unpatentable over Camden (US 6,262,093, 1999) in view of Perdoma *et al.* (J. Cancer Res. Clin. Oncol. 1998, 124, 10-18) in further view of Delatour *et al.* (IDS, Therapie 1976; 31 (4); 505-515).

Camden in view of Perdoma *et al.* teach, as applied to claims 76, 83-97 and 99-106 above, method of treating cancer by inducing apoptosis to a cell expressing abnormal p53 comprising administering a benzimidazole derivative. Moreover, the combination teaches determining the p53 status prior to the administration of a benzimidazole derivative.

Camden in view of Perdoma *et al.* does not teach that the benzimidazole derivative is mebendazole.

Delatour *et al.* teach the embryotoxic and antimitotic properties of benzimidazole compounds (title). Specifically, the reference discloses that in mice with Ehrlich carcinoma mebendazole inhibited tumor growth and increased survival time (abstract).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to include mebendazole as taught by Delatour *et al.* in the method taught by Camden in view of Perdoma. One would have been motivated to make these modifications because as evidenced by Delatour *et al.*, benzimidazole derivatives such as mebendazole have been shown to inhibit tumor growth. Thus, one of ordinary skill in the art would have a reasonable expectation of success that using mebendazole as taught by Delatour *et al.* in the method taught by Camden in view of Perdoma, one would achieve an additional benzimidazole derivative that induces apoptosis in cells and tumors expressing abnormal p53.

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New Rejections upon Reconsideration:

It is noted that Applicants have brought to the Examiner's attention that cancelled dependent claims 22 and 100 were never included in the previous rejection as being anticipated by or obvious over "Camden II" alone or in combination with any of the references cited. However, the claims that further depended from cancelled claims 22 and 100 were included. As such, it appears that the Examiner mistakenly did not include cancelled claims 22 and 100, and therefore, the "new" rejections are set forth below.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 2, 10, 15-19, 21-29, 76, 83, 85, 88-97 and 100-106 are rejected under 35 U.S.C. 103(a) as being unpatentable over Camden (US 5,880,144, 1999) as evidenced by Camden (US Patent 6,262,093, 1999) in view of Perdoma et al. (J. Cancer Res. Clin. Oncol. 1998, 124, 10-18) *of record*.

Camden teaches a method of killing lung tumor cells (A-549), breast tumor cells (MCF-7) and colon tumor cells comprising administering a benzimidazole derivative (column 6, lines 64 to 67, and column 7, Table 3). The patent further teaches a method of treating a patient having cancer comprising administering an effective amount of a benzimidazole derivative to inhibit the growth of the cancer (abstract). With regards to administration, Camden teaches (column 5, lines 1-10) that the benzimidazole derivatives can be administered orally, by intravenous injection, by parental administration or by injection into or around the tumor. Although Camden does not specifically teach that the administration of benzimidazole induces apoptosis, the claimed functional limitation would be an inherent property of the referenced method because as evidenced by Camden (US Patent 6,262,093, 1999), the administration of benzimidazole derivatives results in apoptosis (see column 11, line 65 to column 12, line 51). Thus, it does not appear that the claim language or limitation results in a manipulative difference in the method steps when compared to the prior art disclosure. See Bristol-Myers Squibb Company v. Ben Venue Laboratories 58 USPQ2d 1508 (CAFC

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2001). Moreover, while Camden does not explicitly characterize the tumor cell lines as expressing a tumor suppressor gene such as p53, the claimed functional limitation would be an inherent property of the referenced method since the specification discusses (page 64, Table 4) that A459 tumor cells express wild-type p53. Thus, it does not appear that the claim language or limitation results in a manipulative difference in the method steps when compared to the prior art disclosure. See Bristol-Myers Squibb Company v. Ben Venue Laboratories 58 USPQ2d 1508 (CAFC 2001).

Hence, even though the claims are drawn to a mechanism by cancer cells are inhibited, the claimed method does not appear to distinguish over the prior art teaching of the same or nearly the same method. The mechanism of action does not have a bearing on the patentability of the invention if the invention was already known or obvious. Mere recognition of latent properties in the prior art does not render nonobvious an otherwise known invention. In re Wiseman, 201 USPQ 658 (CCPA 1979). Granting a patent on the discovery of an unknown but inherent function would remove from the public that which is in the public domain by virtue of its inclusion in, or obviousness from, the prior art. In re Baxter Travenol Labs, 21 USPQ2d 1281 (Fed. Cir. 1991). See M.P.E.P. 2145.

Camden does not teach determining the tumor suppressor status by way of Southern blotting, Northern blotting, PCR, ELISA or Western blotting (claims 23-28 and 101-106).

Perdoma *et al.* teach determining the p53 status, by Western blot analysis (page 12, 3rd paragraph) or other methods such as polymerase chain reaction (PCR), could make it possible to predict the response to therapy in certain patients (page 17, 1st column, 2nd paragraph). Perdoma *et al.* further teach that the response to cisplatin *in vivo* of NSCLC tumor lines was dependent on p53 status (page 17, 1st column, 2nd paragraph). Specifically, the reference teaches wt-p53 tumors showed a regression in size of around 60%, whereas mt-p53 tumors stopped growing (page 17, 1st column, 2nd paragraph).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to determine the status of a tumor suppressor gene, like p53, in a tumor cell prior to administering a benzimidazole derivative using techniques such as Western blot, PCR or other methods of analysis. One would have been motivated to do so because Camden teaches the selectivity in killing p53 abnormal cell lines versus cells expressing normal p53 (column 12, lines 52+), while Perdoma *et al.* teaches that the “response to cisplatin *in vivo* of tumors derived from

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different NSCLC lines was dependent on p53 status (page 17, 1st column, 2nd paragraph).” Further, one of ordinary skill in the art would have a reasonable expectation of success because Perdoma *et al.* teaches “analysis of p53 status, by immunohistochemical or other methods such as the polymerase chain reaction (PCR), could make it possible to predict the response to therapy in certain patients (page 17, 1st column, 2nd paragraph).”

Claims 3 and 77 are rejected under 35 U.S.C. 103(a) as being unpatentable over Camden (US 5,880,144, 1999) in view of Perdoma *et al.* (J. Cancer Res. Clin. Oncol. 1998, 124, 10-18) *of record* in further view of either of Delatour *et al.* (IDS, Therapie 1976; 31 (4); 505-515) *of record* or Nasr *et al.* (Journal of Pharmaceutical Sciences 1985; 74: 831-836).

Camden in view of Perdoma *et al.* teach, as set forth above for claims 2, 10, 15-19, 21-29, 76, 83, 85, 88-97 and 100-106, a method of killing lung tumor cells (A-549), breast tumor cells (MCF-7) and colon tumor cells comprising administering a benzimidazole derivative (column 6, lines 64 to 67, and column 7, Table 3). The patent further teaches a method of treating a patient having cancer comprising administering an effective amount of a benzimidazole derivative to inhibit the growth of the cancer (abstract). Moreover, the combination teaches determining the p53 status prior to the administration of a benzimidazole derivative.

Camden in view of Perdoma *et al.* does not teach that the benzimidazole derivative is mebendazole.

Delatour *et al.* teach the embryotoxic and antimitotic properties of benzimidazole compounds (title). Specifically, the reference discloses that a method of inhibiting tumor growth in mice comprising administering the benzimidazole derivative, mebendazole (abstract).

Nasr *et al.* teach (page 831, paragraph bridging 1st column and 2nd) *in vivo* anticancer activity correlation of aromatic, aliphatic, and heterocyclic carbamates and their thio-isosters against both intraperitoneally implanted murine P-388 lymphocytic leukemia and L-1210 lymphoid leukemia. Specifically, the reference teaches anticancer activity of benzimidazole carbonates (page 834, Table VIII and page 835, 2nd column, 2nd full paragraph).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to combine the references so as to inhibit tumor growth because each of the benzimidazole derivatives disclosed by the references have close structural similarities and similar

utilities. In the instant case, the courts have held that "An obviousness rejection based on similarity in chemical structure and function entails the motivation of one skilled in the art to make a claimed compound, in the expectation that compounds similar in structure will have similar properties." In *re Payne*, 606 F.2d 303, 313, 203 USPQ 245, 254 (CCPA 1979). See *In re Papesch*, 315 F.2d 381, 137 USPQ 43 (CCPA 1963); *In re Dillon*, 919 F.2d 688, 16 USPQ2d 1897 (Fed. Cir. 1991) (see in MPEP § 2144) for an extensive review of the case law pertaining to obviousness based on close structural similarity of chemical compounds. See also MPEP § 2144.08, paragraph II.A.4.(c). Thus, one of skill in the art would have a reasonable expectation of success that by substituting a benzimidazole derivate as taught by Delatour et al. or Nasr et al. in the method of Camden in view of Perdoma et al., one would achieve a method of inhibiting the growth of cancer.

Claims 13-14 and 86-87 are rejected under 35 U.S.C. 103(a) as being unpatentable over Camden (US 5,880,144, 1999) in view of Perdoma et al. (J. Cancer Res. Clin. Oncol. 1998, 124, 10-18) *of record* in further view of Lucci et al. (Cancer; 86:300-311, published online on November 2000).

Camden in view of Perdoma et al. teach, as set forth above for claims 2, 10, 15-19, 21-29, 76, 83, 85, 88-97 and 100-106, a method of killing lung tumor cells (A-549), breast tumor cells (MCF-7) and colon tumor cells comprising administering a benzimidazole derivative (column 6, lines 64 to 67, and column 7, Table 3). The patent further teaches a method of treating a patient having cancer comprising administering an effective amount of a benzimidazole derivative to inhibit the growth of the cancer (abstract). Moreover, the combination teaches determining the p53 status prior to the administration of a benzimidazole derivative.

Camden in view of Perdoma et al not teach that the tumor cell is a multidrug resistant tumor cell, wherein the tumor cell is a breast tumor cell.

Lucci et al. teach multidrug resistance modulators and doxorubicin synergize to elevate ceramide levels and elicit apoptosis in drug-resistant cancer cells, specifically drug resistant human breast cancer cells lines. Moreover, the reference teaches that multidrug resistance is a formidable roadblock to the effective treatment of cancer by conventional chemotherapy, wherein the resistance complicates treatment in many instances (page 300, 1st paragraph).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to use a multidrug resistant cell line, such as a breast cancer cell, in the method

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taught by Camden in view of the teachings of Lucci et al. One would have been motivated to do so because as taught by Lucci, multidrug resistance is a formidable roadblock to the effective treatment of cancer by conventional chemotherapy, wherein the resistance complicates treatment in many instances. Thus, one of ordinary skill in the art would have a reasonable expectation of success that by administering a benzimidazole derivative to multidrug resistant cell, one would achieve a method of inhibiting tumor growth in a patient that has already become resistant to conventional chemotherapy.

Note: Claim 20 and 98 are objected to as being dependent from a rejected independent claim.

All other rejections and/or objections are withdrawn in view of applicant's amendments and arguments there to.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Brandon J. Fetterolf, PhD whose telephone number is (571)-272-2919. The examiner can normally be reached on Monday through Friday from 7:30 to 4:30.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Jeff Siew can be reached on 571-272-0787. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Brandon J Fetterolf, PhD
Patent Examiner
Art Unit 1642


JEFFREY SIEW
SUPERVISORY PATENT EXAMINER



Notice of References Cited	Application/Control No. 10/043,877	Applicant(s)/Patent Under Reexamination MUKHOPADHYAY ET AL.	
	Examiner Brandon J. Fetterolf, PhD	Art Unit 1642	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-6,262,093	07-2001	Camden, James Berger	514/365
*	B	US-5,880,144	03-1999	Camden, James Berger	514/397
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
*	U	Perdoma et al. (J. Cancer Res. Clin. Oncol. 1998, 124, 10-18)
*	V	Delatour et al. (Therapie 1976; 31 (4); 505-515)
*	W	Nasr et al. (Journal of Pharmaceutical Sciences 1985; 74: 831-836)
*	X	Lucci et al. (Cancer; 86:300-311, published online on November 2000).

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

EXHIBIT 7

P. Delatour¹, Y. Richard¹ Embryogenic and antimitotic properties of benzimidazole series
Thérapie, 1976, 31:505-515

¹ Toxicology and Virology Laboratories, National Veterinary School of Lyon, 2, quai
Chauveau, 69337 Lyon Cedex 1

Text sent December 22, 1975, accepted March 30, 1976

Reprints: P. Delatour, same address

EMBRYOTOXIC AND ANTIMITOTIC PROPERTIES OF BENZIMIDAZOLE SERIES

//insert English summary, p. 505//

Whereas in general, drugs designed for animal therapy contain active principles used in addition in human medicine, it is a pharmacological class, that of anthelmintics, where for economic reasons, veterinary research is more active and gives rise to original compounds. The discovery of thiabendazole (MSD) widely used as much for fungicide as anthelmintic, inspired various research studies in the benzimidazole series which has resulted in numerous new drugs in recent years.

EMBRYOTOXIC PROPERTIES

Our attention was initially drawn to this chemical family because of teratological effects observed in the sheep following the administration of parbendazole DC [common name] [5,12]. The confirmation of these facts was later reported in the same species [16,17,18] while we were experimentally expanding this idea of teratogenesis with cambendazole in the ewe [7]. The goal of the experiments that are conducted for theoretical and practical reasons at the same time in the rat is firstly, to confirm on a laboratory species the clinical phenomena observed as well to start a possible structure/activity relationship. Secondly, within the perspective of the recent law of Veterinary Pharmacy (J.O. [Official Journal], May 30, 1975), the opportunity was offered to test the possible projected interest in experimentations on a laboratory animal with a view to forming the files required to obtain the marketing authorization.

Table I – Chemical constitution

//insert, p. 506, new keyed copy//

Key: 1 Compounds
 2 M.W.
 3 Carbendazim
 4 Mebendazole

Material and methods

The compounds included in this study are either products currently marketed in France or samples given out by industrial firms, or lastly original molecules synthesized for this purpose (Prof. Daudon, U.E.R. of Pharmaceutical Sciences, Lyon). All are derivatives of benzimidazole whose list appears in Table I.

The experimental protocols of the study of embryotoxic properties, detailed earlier [6],

On the 21st day, after sacrificing the rats, counting of the implantations and full-term fetuses are carried out. After individually weighing the latter, an inventory of external skeletal and visceral malformations is established.

Results

The most active compounds of this series are at the same time embryolethal, teratogenic and fetotoxic according to the general rule of embryo-fetal toxicology. In this case the dose-effect is very clear since, with increase in the daily dosage, one simultaneously witnesses an increase in embryonic mortalities, an increase in the percentage of subjects afflicted with malformations, and a decrease in average full-term fetus weight (Table II, Figure 1). The malformations observed are practically identical from one compound to the other (exencephaly, meningocele, hydrocephaly, harelip, microanophthalmia, hypodysplasia of limbs, ectrodactyly, microanuria). However, the frequency of a given abnormality varies, often significantly, according to the compound considered. Thus, for example hydrocephaly, very frequent with MBDZ, is more rare with PBDZ and CBDZ ($p = 0.05$). Exencephaly, very frequent with BCM and PBDZ, is more occasional otherwise. Likewise, harelip is much more frequent ($p = 0.01$) with CyBDZ and MBDZ than with the rest of the chemical family. Lastly, combined with external malformations, there are numerous skeletal abnormalities (costal fusing and bifurcations, fusion or absence of body and vertebral arches as well as sternbrae, defects in ossification of the bone of the cranium and limbs) and visceral (cleft palates, hydronephrosis).

Table II – Embryotoxicity in the SD rat
//insert, p. 508//

Key:	1	Compounds
	2	Oral dose
	3	Number of rats
	4	Embryolethality
	5	External abnormalities
	6	Fetus weight
	7	Control

//insert, p. 509//

Figure 1 – Embryolethality of substituted 2/5 benzimidazolyl compounds in the rat

Key: 1 [Illegible]

ANTIMITOTIC PROPERTIES

Various arguments taken from the literature in unpublished documents or in the experimental results reported above led to testing the possible antimitotic effects of some of these compounds. In particular, the following facts were retained:

- Existence of a powerful teratogenic power in the mouse and the rat. Furthermore, in this latter species, some malformations by their nature are reminiscent of those produced by vincalkeboblaline [3] and actinomycin D [19]:

- Production of alopecia in the rat and loss of wool in the sheep [19]; phenomenon also known with cyclophosphamide [9];

- Inhibition of spermatogenesis and hematopoeisis in the rat during chronic administration.

Material and methods

The in vitro tests on leukocytes were carried out according to a technique by Carpentier et al. [2]. The blood is incubated for 72 h in medium 199 with added phytohemagglutinin, then put in contact with the products being studied for 4 h at a concentration of 1 $\mu\text{g/mL}$. Then they are fixed, and the nuclei stained with Giemsa, then the mitotic index is determined by counting at least 1000 nuclei with the microscope [8]. The cell cultures are carried out with primary explants of the pig kidney or continuous HeLa, BHK/21 or KB lines in Eagle medium with the addition of lactalbumin hydrolyzate and complement-inactivated calf serum. The cell suspension (10^5 cells/mL) is distributed into Leighton tubes then held in the incubator for 24 to 36 h. The medium is then replaced by a medium containing the products studied at a concentration of 2 $\mu\text{g/mL}$. At the end of contact times of 2, 4, 6, 12, 24 or 48 h, they are fixed then stained with May-Grunwald-Giemsa [3,15]. The graft of Ehrlich's carcinoma to the Swiss male mouse is carried out by ip injection of 0.5 mL of a liquid pool of ascites kept alive by passages sampled the 3rd day of development on animals. The treatments, administered orally in one (x) or two (x + x) times per 24 h are started the day following the day of the graft and continued for 8 consecutive days. The ratio of the tumor mass T(n) to Po (body weight of animals on the day of the graft) was determined daily; the average survival duration is calculated.

Results

On human leukocytes, the mitotic index is established as follows:

(X: significantly different result (χ^2) from the control:

//insert table, mid 510//

Key: 1 Control
2 MI

A similar phenomenon of increase in MI is observed in sheep leukocytes:

//insert second little table,510//

Key: 1 Control
2 MI

In cell cultures, all the lines maintained do not offer the same sensitivity to the cytotoxic activity of the compounds: the primary explant is the most sensitive, the KB cell the least. On the other hand, the nature of the cytological observations observed is comparable or identical from one biological material to the other. There is noted:

- an inhibition of cell proliferation that is all the more intense when the duration of exposure to the product is extended longer (Table III);
- a rise in the mitotic index that is attributable to the appearance of abnormal mitoses (stathmokinesis [pseudometaphase]) as well as the number of pyknotic nuclei (Figure 2);
- the existence of multinucleate cells, cells with multilobed nuclei or giant polyploid nuclei.

Table III – Effect of N-benzimidazolyl 2/5 carbamates on KB cell cultures.

//insert, p. 511//

CP: cell proliferation expressed by the average number of cells per microscopic field

MI: mitotic index determined on a minimum of 1000 nuclei

(*) and (**): results significantly different from the control lot (χ^2) with the risk of 5% and 1%, respectively.

Key:	1	Compounds
	2	2 h contact
	3	4 h contact
	4	6 contact
	5	Control
	6	CP
	7	MI

//insert, p. 512//

Figure 2 – Effects of CBDZ on primary explant of pig kidney

On the left: control 16-h culture. Mitoses: 21/1000. Pyknotic nuclei: 14/1000

On the right: free 12-h culture: then CBDZ 2 μ g/mL for 4 h. Mitoses: 89/1000. Pyknotic nuclei: 40/1000

Table IV – Effects on the development of Ehrlich's carcinoma of the Swiss mouse

//insert table, p. 512//

(*) Significantly different (F test) from the control with risk of 5%

(**) Subcutaneous administration

Key: 1 Compounds
 2 Oral daily dose
 3 Number of mice
 4 Average survival
 5 Control

DISCUSSION

From the embryotoxic point of view, if the base nuclei (BX, TBDZ) are inactive, almost all embryo-lethal and teratogenic compounds are N-benzimidazolyl-2 carbamates (PBDZ, MBDZ, CyBDZ) or N-benzimidazolyl-5 carbamates (CBDZ, CBDZ(Me), CBDZ-NH₂). However, the carbamate group does not seem to be indispensable since a ureine presents a similar activity compared to BCM and BCM(NH₂). The corresponding primary amines, lacking the

benzimidazolyl-2. With the series of N-benzimidazolyl-2 carbamates, the embryotoxicity of the various representatives is extremely variable. The substitution at C(5) is not indispensable since BCM is active. On the other hand, according to the nature of the substituent, it goes from very active molecules to nontoxic compounds under the same experimental conditions. This fact is all the more striking since the substituting residues may be chemically very close (compare: MBDZ and FBDZ, PBDZ and OBDZ).

From the antimitotic point of view, it is noted that for most of the compounds considered, the correlation between in vivo and in vitro activity is high since the very cytostatic molecules on the leukocytes as well as on the KB cells (PBDZ, MBDZ, CBDZ, CyBDZ) are also cancer-suppressing on Ehrlich's tumor; conversely OFDZ and DD-73 are inactive on both types of experimental models. However several differences are to be noted, the most obvious being related to BCM and OBDZ, both active in vitro but totally lacking in activity on the carcinoma of the mouse. The possible connection between embryotoxic activity and anticancer activity is verified in the majority of cases as much among inactive compounds (FBDZ, DD-73, OBDZ, BZ·NH·Ac) as among active compounds (PBDZ, MBDZ, CyBDZ, CBDZ). Metabolic considerations would make it possible to clarify without a doubt among the N-benzimidazolyl-carbamates, certain differences attributable to the change in animal species (embryotoxic BCM in the rat but not anticancer in the mouse) or to the route of administration (OBDZ inactive on Ehrlich's carcinoma orally but inactive subcutaneously). Some of these compounds are also active on L-1210 leukemia of the DBA/2 and the BDF-1 mouse [8,11] on spontaneous leukemia in the AKR mouse [13] as well as on P-388 leukemia. Lastly, PBDZ and CBDZ seem endowed with favorable effects on spontaneous lymphoid leukosis in the dog. Moreover, a derivative of N-benzimidazolyl-2 carbamate not appearing in the list above and close to MBDZ has been found to be active on B16 melanocarcinoma and Lewis lung cancer in the mouse [1]. The nature of the cytotoxic effects observed in vitro on cell cultures make it possible to think that the mode of action of these compounds on the animal cell is identical to that of BCM on *Aspergillus nidulans* [4]. It would consist of a disorganization of microtubules of the mitotic spindle and would therefore be comparable to that of colchicines and vinblastine.

The consequences of these experimental results are in various orders. From the point of

December 27, 1974) and which could be extended to MBDZ. From the medical point of view, PBDZ, which within this chemical series offers the best activity with regard to its tolerance, has been the subject of clinical trials in man (Prof. Cl. Lapras, Neurological Hospital, Lyon). The value of this molecule seems to be confirmed on neoblastomas and glioblastomas during oral administration, local infiltration or intrathecal injection [14]. Moreover, these facts bring up new antimitotic structures. A similar reasoning to ours inspired overseas the experimental and clinical study of anticancer properties of the very close compound which is N((thienyl-2 carbonyl)5, benzimidazolyl)2, methyl carbamate [1] initially patented as anthelmintic [20].

The continuation of these investigations, oriented by the structure/activity relationship, could make it possible to discover new worthwhile therapeutic molecules.

Bibliography

//insert 1//

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//insert 3-4//

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//insert 16, 17, 18//

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Propriétés embryotoxiques et antimitotiques en série benzimidazole

P. DELATOUR, Y. RICHARD

Laboratoires de Toxicologie et de Virologie, Ecole Nationale Vétérinaire de Lyon,
2, quai Chauveau, 69337 Lyon Cedex 1

RÉSUMÉ. — La découverte des propriétés tératogènes d'un anthelminthique dérivé du benzimidazole chez l'espèce ovine dans les conditions thérapeutiques a incité à étudier au Laboratoire les propriétés biologiques d'une série de 24 dérivés de cette famille chimique. Certains de ces composés sont doués d'effets embryotoxiques intenses chez le rat et la souris. Ce phénomène est expliqué par la mise en évidence de propriétés antimitotiques démontrées sur différents modèles expérimentaux *in vitro* et *in vivo*. Ces faits, confirmés pour l'un d'eux en Clinique humaine, suggèrent de nouvelles structures potentiellement anticancéreuses.

SUMMARY. — The discovery of the teratogenic properties of the anthelmintic drug parbendazole C.D. under therapeutical conditions in the ewe has well urged to study the biological effects of 24 benzimidazole-related compounds. Some of them (N-benzimidazolyl-2, carbamates and N-benzimidazolyl-5, carbamates) are highly teratogenic in the rat. They have also an antimitotic activity *in vitro* (human and ovine leukocytes, primary explant of pig kidney, HeLa, BHK/21 and KB cells) and *in vivo* (Ehrlich carcinoma in the Swiss mouse, L-1210 leukemia in the DBA/2 mouse, spontaneous leukemia of the AKR mouse and P-388 leukemia). The therapeutical interest of one of them seems to be confirmed upon some nervous tumors of the man. The analysis of the structure-activity relationship suggests some new anticancerous compounds.

Si, en général, les médicaments destinés à la thérapeutique animale renferment des principes actifs utilisés, par ailleurs, en Médecine Humaine, il est une classe pharmacologique — celle des anthelminthiques — où, pour des raisons économiques, la recherche vétérinaire est plus active et donne naissance à des composés originaux. La découverte du thiabendazole (MSD), largement utilisé tant comme fongicide que comme anthelminthique, a inspiré diverses recherches dans la série du benzimidazole qui ont abouti ces dernières années à de nombreux médicaments nouveaux.

Texte adressé le 22 décembre 1975, accepté le 30 mars 1976.
Tirés-à-part : P. DELATOUR, même adresse.

PROPRIETES EMBRYOTOXIQUES

Notre attention fut initialement attirée sur cette famille chimique en raison d'accidents tératologiques observés dans l'espèce ovine faisant suite à l'administration de parbendazole DC [5, 12]. La confirmation de ces faits fut ultérieurement rapportée dans la même espèce [16, 17, 18], tandis que nous étendions expérimentalement chez la brebis cette notion de tératogénèse au cambendazole [7]. Le but des expérimentations qui sont conduites pour des raisons à la fois théoriques et pratiques chez le Rat est, dans un premier temps de confirmer sur une espèce de laboratoire les phénomènes observés en clinique ainsi que de dégager une éventuelle relation structure/activité. En second lieu, dans la perspective de la récente loi sur la Pharmacie Vétérinaire (J. O. du 30-5-75), l'occasion s'offrait de tester l'éventuel intérêt prévisionnel des expérimentations sur animal de laboratoire en vue de la constitution des dossiers requis pour l'obtention des autorisations de mise sur le marché.

TABLEAU I. — Constitutions chimiques.

Composés	R (2)	R' (5)	R'' (3)	p. m
benzimidazole BZ	—H	H—	H	106
BZ—NH ₂	—NH ₂	H—	H	121
BZ.NH.Ac	—NH—CO—CH ₃	H—	H	175
carbendazime BCM	—NH—CO—O—CH ₃	H—	H	191
OH—BCM	—NH—CO—O—CH ₃	OH—	H	207
BCM (NH)	—NH—CO—NH—CH ₃	H—	H	190
parbendazole PBDZ	—NH—CO—O—CH ₃	CH ₃ CH ₂ CH ₂ CH ₂ —	H	247
PBDZ (NH ₂)	—NH ₂	CH ₃ CH ₂ CH ₂ CH ₂ —	H	189
mébendazole MBDZ	—NH—CO—O—CH ₃	C ₆ H ₅ —CO—	H	295
MBDZ (NH ₂)	—NH ₂	C ₆ H ₅ —CO—	H	238
MBDZ.H ₂	—NH—CO—O—CH ₃	C ₆ H ₅ —CHOH—	H	297
fenbendazole FBDZ	—NH—CO—O—CH ₃	C ₆ H ₅ —S—	H	299
oxfendazole OFDZ	—NH—CO—O—CH ₃	C ₆ H ₅ —SO—	H	315
CyBDZ	—NH—CO—O—CH ₃	(CH ₂) ₂ =CH—CO—	H	259
DD-73	—NH—CO—O—CH ₃	CH ₃ —O—CO—NH—	H	264
oxibendazole OBDZ	—NH—CO—O—CH ₃	CH ₃ CH ₂ CH ₂ —O—	H	249
Benomyl BNM	—NH—CO—O—CH ₃	H—	—CO—NH—n Bu	290
thiabendazole TBDZ	thiazolyl-4	H—	H	201
TBDZ—NH ₂	thiazolyl-4	NH ₂ —	H	216
TBDZ—NH—Ac	thiazolyl-4	CH ₃ —CO—NH—	H	258
CBDZ (Me)	thiazolyl-4	CH ₃ —O—CO—NH—	H	274
TBDZ—NH—Bz	thiazolyl-4	C ₆ H ₅ —CO—NH—	H	320
cambendazole CBDZ	thiazolyl-4	iPr—O—CO—NH—	H	302
CBDZ—NH ₂	thiazolyl-4	iPr—O—CO—NH ₂ —	—NH ₂	317

Matériel et Méthodes.

Les composés inclus dans cette étude sont soit des produits actuellement commercialisés en France, soit des échantillons cédés par des industriels, soit enfin des molécules originales synthétisées à cet effet (Pr DAUDON, U.E.R. des Sciences Pharmaceutiques, Lyon). Tous sont des dérivés du benzimidazole dont la liste figure au tableau I.

Les protocoles expérimentaux de l'étude des propriétés embryotoxiques, détaillée antérieurement [6], répondent aux normes habituellement admises. À noter qu'en raison de la grande embryotoxicité de ces composés, l'administration est réalisée du 8^e au 15^e jour de gestation. De plus, afin de faire abstraction des différences de poids moléculaires d'un composé à l'autre (BZ pm = 106 ; CBDZ.NH₂ pm = 317) les doses administrées sont des fractions ou multiples de D :

$$D = 10^{-4} \text{ Mole/kg}$$

Le 21^e jour, après sacrifice des rates, on procède au dénombrement des nida-tions et des fœtus à terme. Après pesée individuelle de ces derniers, l'inventaire est alors établi des malformations externes squelettiques et viscérales.

Résultats.

Les composés les plus actifs de cette série sont à la fois embryo-léthaux, tératogènes et fœtotoxiques selon la règle générale en toxicologie embryo-fœtale. Dans ce cas, l'effet-dose est très net puisque avec l'élévation de la posologie quotidienne, on assiste simultanément à l'augmentation des mortalités embryonnaires, à l'élévation du pourcentage de sujets atteints de malformations et à la diminution du poids moyen des fœtus à terme (*tableau II, figure 1*). Les malformations observées sont pratiquement identiques d'un composé à l'autre (exencéphalies, ménin-gocèles, hydrocéphalies, becs-de-lièvre, micro-anophtalmies, hypo-dysplasies des membres, ectrodactylies, micro-anouries). Cependant, la fréquence d'une anomalie donnée varie, souvent significativement, selon le composé envisagé. Ainsi par exemple, l'hydro-céphalie, très fréquente avec MBDZ, est plus rare avec PBDZ et CBDZ ($p = 0,05$). L'exencéphalie, très fréquente avec BCM et PBDZ, est plus occasionnelle par ailleurs. De même, le bec-de-lièvre est beaucoup plus fréquent ($P = 0,01$) avec CyBDZ et MBDZ que dans le reste de la famille chimique. Enfin, associées aux malformations externes, existent de nombreuses anomalies squelettiques (soudures et bifurcations costales, fusion ou absence de corps et d'arcs vertébraux ainsi que de sternites, défauts d'ossification des os du crâne et des membres) et viscérales (fentes palatines, hydronéphrose).

PROPRIETES ANTIMITOTIQUES

Divers arguments puisés dans la littérature, dans des documents non publiés ou dans les résultats expérimentaux rapportés ci-dessus invitaient à tester les éventuels effets antimitotiques de certains de ces composés. Ont, en particulier, été retenus les faits suivants :

TABLEAU II. — Embryotoxicité chez le Rat S.D.

Composés	Dose orale D = mg/kg	Nombre de rates	Embryo- léthalité	Anomalies externes	Poids fœtus
Témoin		40	5,3 %	0,2 %	3,7 g
BZ	4D = 47,2	6	2,9	0	3,7
BZ.NH ₂	2D = 26,6	3	5,0	0	4,0
	4D = 53,2	2	25,9 (**)	0	4,4
BZ.NH.Ac	D = 17,5	4	6,5	0	3,45(*)
	2D = 35,0	4	17,2 (*)	0	3,5 (*)
BCM	D/2 = 9,55	4	2,0	0	3,7
	D = 19,1	7	72,5 (**)	100 (**)	2,1 (**)
	2D = 38,2	2	100 (**)		
BCM(NH)	D = 19,0	8	65,7 (**)	6,25 (*)	2,7 (*)
	3D = 57,0	4	100 (**)		
BNM	4D = 116,0	7	1,1	0	3,6
PBDZ	2D/5 = 10	9	21,2 (**)	16,9 (**)	3,0
	D = 24,7	11	61,7 (**)	51,0 (**)	2,5 (**)
	8D/5 = 40	15	99,0 (**)	100 (**)	1,6 (**)
PBDZ(NH ₂)	D = 18,9	4	8,9	0	3,7
	4D = 75,6	4	20,45(**)	0	2,9 (*)
MBDZ	D/3 = 9,8	10	15,2 (**)	4,2 (*)	3,15(*)
	D/2 = 14,7	11	88,0 (**)	100 (**)	3,0 (**)
	D = 29,5	7	88,2 (**)	100 (**)	2,4 (**)
MBDZ(NH ₂)	4D = 94,4	4	21,9 (**)	0	2,9 (*)
CyBDZ	D/3 = 8,6	3	35,3 (**)	0	3,4
	D/2 = 12,95	4	39,5 (**)	45,0 (**)	2,7 (*)
	2D/3 = 17,3	4	83,7 (**)	71,4 (**)	2,6 (*)
OBDZ	D = 24,9	10	6,6	0	3,7
	6D = 149,4	8	9,8	0	3,45(*)
FBDZ	2D = 59,8	4	3,45	0	
	4D = 119,6	4	3,8	0	3,8
MBDZ.H ₂	D/4 = 7,4	4	32,1 (**)	5,4 (*)	2,8 (*)
	D/3 = 9,9	3	70,6 (**)	100 (**)	2,5 (*)
	D/2 = 14,8	3	100 (**)		
DD-73	D/3 = 8,8	4	6,8	0	4,3
	D = 26,4	4	10,0	0	3,9
TBDZ	4D = 80,8	8	6,1	0	3,6
TBDZ.NH ₂	D/3 = 7,2	4	3,7	0	3,7
	2D = 43,4	3	4,9	0	4,0
TBDZ.NH.Ac	D/3 = 8,6	4	18,7 (*)	0	3,6
	D = 25,8	4	3,7	0	3,9
TBDZ.NH.Bz	D/3 = 10,3	3	2,3	0	3,5
CBDZ(Me)	D/2 = 13,8	6	16,5 (**)	0	3,1 (*)
	2D/3 = 18,4	5	57,9 (**)	43,75(**)	2,9 (**)
	D = 27,6	2	100 (**)		
CBDZ	D/4 = 7,6	8	8,9	3,9 (*)	3,25(*)
	D/3 = 10,1	13	75,0 (**)	73,8 (**)	2,1 (**)
	D/2 = 15,15	6	80,7 (**)	100 (**)	1,7 (**)
CBDZ.NH ₂	D/3 = 10,6	4	5,65	0	3,7
	2D/3 = 21,2	4	71,7 (**)	100 (**)	1,9 (**)
	D = 31,8	3	100 (**)		

(*) et (**): résultats significativement différents (Tests X² ou F) du lot témoin aux risques respectifs de 5 p. 100 et de 1 p. 1000.

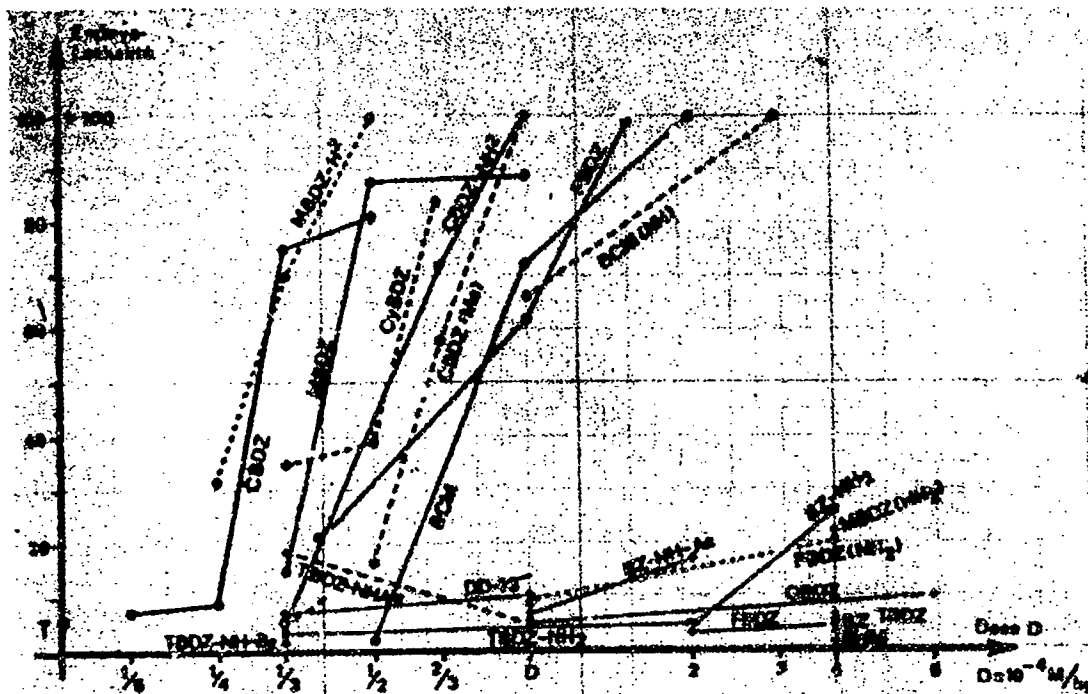


FIG. 1. — Embryoléthalité de composés benzimidazolyl 2/5 substitués chez le Rat.

— existence d'un pouvoir embryoloéthral intense : chez le rat, le rapport de la DL-50 à la dose embryoloéthrale-50 est très élevé :

CBDZ DL-50/DEL-50	= 105
MBDZ	≥ 106
PBDZ	> 300
BCM	> 400

— existence d'un pouvoir tératogène puissant chez la souris et le rat. De plus, chez cette dernière espèce, certaines malformations rappellent par leur nature celles produites par la vincaléukoblastine [3] et l'actinomycine D [19] ;

— production d'alopécies chez le rat et chute de la laine chez le mouton [10] ; phénomène également connu avec le cyclophosphamide [9] ;

— inhibition de la spermatogénèse et de l'hématopoïèse chez le rat lors d'administration chronique.

Matériel et Méthodes.

Les essais *in vitro* sur leucocytes sont réalisés selon une technique de CARPENTIER et coll. [2]. Le sang est incubé pendant 72 heures en milieu 199 additionné de phytohémagglutinine puis mis au contact des produits étudiés pendant 4 heures à la concentration de 1 $\mu\text{g/ml}$. On réalise alors la fixation puis la coloration des noyaux au Giemsa, puis on détermine l'index mitotique par comptage au microscope d'au moins 1.000 noyaux [8]. Les cultures cellulaires sont réalisées sur explants primaires de rein de porc ou lignées continues HeLa, BHK/21 ou KB en milieu de Eagle additionné d'hydrolysate de lactalbumine et de sérum de veau décomplémenté. La suspension cellulaire (10^5 cellules/ml) est distribuée en tubes

de LEIGHTON puis portée à l'étuve pendant 24 à 36 heures. Le milieu est alors remplacé par un milieu contenant les produits étudiés à la concentration de 2 mcg/ml. Au terme de temps de contact de 2, 4, 6, 12, 24 ou 48 heures, on réalise la fixation puis la coloration au MAY-GRUNWALD-GIEMSA [8, 15]. La greffe du carcinome d'EHRICH à la souris Swiss mâle est réalisée par injection I.P. de 0,5 ml d'un pool de liquide d'ascite prélevé le 8^e jour d'évolution sur des animaux entretenus par passages. Les traitements, administrés par voie orale en une (x) ou deux (x + x) fois par 24 heures sont instaurés le lendemain du jour de la greffe et poursuivis pendant 8 jours consécutifs. On détermine quotidiennement le rapport de la masse tumorale T(n) à Po (poids corporel des animaux le jour de la greffe) ; on calcule la durée moyenne de survie.

Résultats.

Sur leucocytes humains, les index mitotiques s'établissent comme suit : (X : résultat significativement différent (X^2) du témoin) :

témoin	I.M. = 44 p. 1.000
OFDZ	= 40
DD-73	= 43
MBDZ	= 92 (X)
BCM(NH)	= 108 (X)
CBDZ(Me)	= 114 (X)
PBDZ	= 116 (X)
CyBDZ	= 120 (X)
FBDZ	= 130 (X)
CBDZ	= 139 (X)
BCM	= 142 (X)
OBDZ	= 149 (X)

Un phénomène analogue d'élévation de I.M. est observé sur leucocytes de mouton :

témoin	I.M. = 34 p. 1.000
CBDZ	= 76 (X)
PBDZ	= 98 (X)

Sur cultures cellulaires, toutes les lignées retenues n'offrent pas la même sensibilité à l'activité cytotoxique de ces composés : l'explant primaire est le plus sensible, la cellule KB l'est le moins. En revanche, la nature des altérations cytologiques observées est comparable ou identique d'un matériel biologique à l'autre. On note :

- une inhibition de la prolifération cellulaire d'autant plus intense que la durée d'exposition au produit est plus prolongée (*tableau III*) ;
- une élévation de l'index mitotique imputable à l'apparition de mitoses anormales (stathmométaphases) ainsi que du nombre des noyaux picnotiques (*fig. 2*) ;
- l'existence de cellules multinucléées, de cellules à noyaux plurilobés ou à noyaux géants polyploïdes.

Les effets sur le carcinome d'EHRICH de la souris Swiss sont indiqués dans le *tableau IV*. On constate que pour certains composés tels que PBDZ,

TABLEAU III. — Effets de N-benzimidazolyl 2/5, carbamates sur cultures de cellules KB.

Composés	T ₀		Contact de 2 h		Contact de 4 h		Contact de 6 h	
	P.C.	I.M.	P.C.	I.M.	P.C.	I.M.	P.C.	I.M.
témoin	95	22	76	25	137	29	204	39
BCM			111	32	150	42	144 (*)	30
OH.BCM			112	37	118	53	200	37
BCM(NH)			89	43 (*)	97	78 (**)	83 (**)	115 (**)
PBDZ			80	40	67 (**)	58 (**)	97 (**)	157 (**)
OBDZ			89	66 (**)	92 (*)	61 (**)	46 (**)	203 (**)
FBDZ			125	51 (*)	169	67 (**)	94 (**)	87 (**)
MBDZ			106	56 (**)	139	44	84 (**)	130 (**)
OFDZ			103	40	145	41	109 (**)	42
MBDZ.H ₂			85	38	115	94 (**)	133 (*)	168 (**)
CyBDZ			104	70 (**)	124	121 (**)	139 (*)	180 (**)
DD-73			72	35	132	35	166	46
CBDZ			89	45 (*)	113	97 (**)	78 (**)	123 (**)
CBDZ(Me)			90	59 (**)	97	63 (**)	68 (**)	97 (**)
CBDZ.NH ₂			80	35	89 (*)	48 (*)	110 (**)	91 (**)

P.C. : prolifération cellulaire exprimée par le nombre moyen de cellules par champs microscopiques.

I.M. : index mitotique déterminé sur un minimum de 1.000 noyaux.

(*) et (**): résultats significativement différents du lot témoin (X²) aux risques respectifs de 5 p. 100 et de 1 p. 1.000.

MBDZ et CBDZ, il est noté à la fois un ralentissement de l'évolution du processus tumoral et une augmentation de la survie des animaux. Pour BCM, BCM(NH), OBDZ administré oralement, FBDZ, OFDZ, DD-73 et BZ.NH₂.Ac, l'activité anticancéreuse paraît nulle en dépit d'une bonne tolérance générale dans les conditions de posologie indiquées.

DISCUSSION

Du point de vue embryotoxique, si les noyaux de base (BZ, TBDZ) sont inactifs, la quasi-totalité des composés embryoléthaux et tératogènes sont des N-benzimidazolyl-2, carbamates (PBDZ, MBDZ, CyBDZ) ou des N-benzimidazolyl-5, carbamates (CBDZ, CBDZ(Me), CBDZ-NH₂). Le groupement carbamate ne semble cependant pas indispensable puisqu'une uréine présente une activité analogue : comparer BCM et BCM(NH). Les amines primaires correspondantes, dépourvues du groupement carbonyle, ne sont pas tératogènes et ne sont modérément embryotoxiques (20 à 25 p. 100) qu'à doses très élevées, proches de la toxicité maternelle (comparer : PBDZ et PBDZ(NH₂), MBDZ et MBDZ(NH₂), BCM et BZ-NH₂, CBDZ et TBDZ-

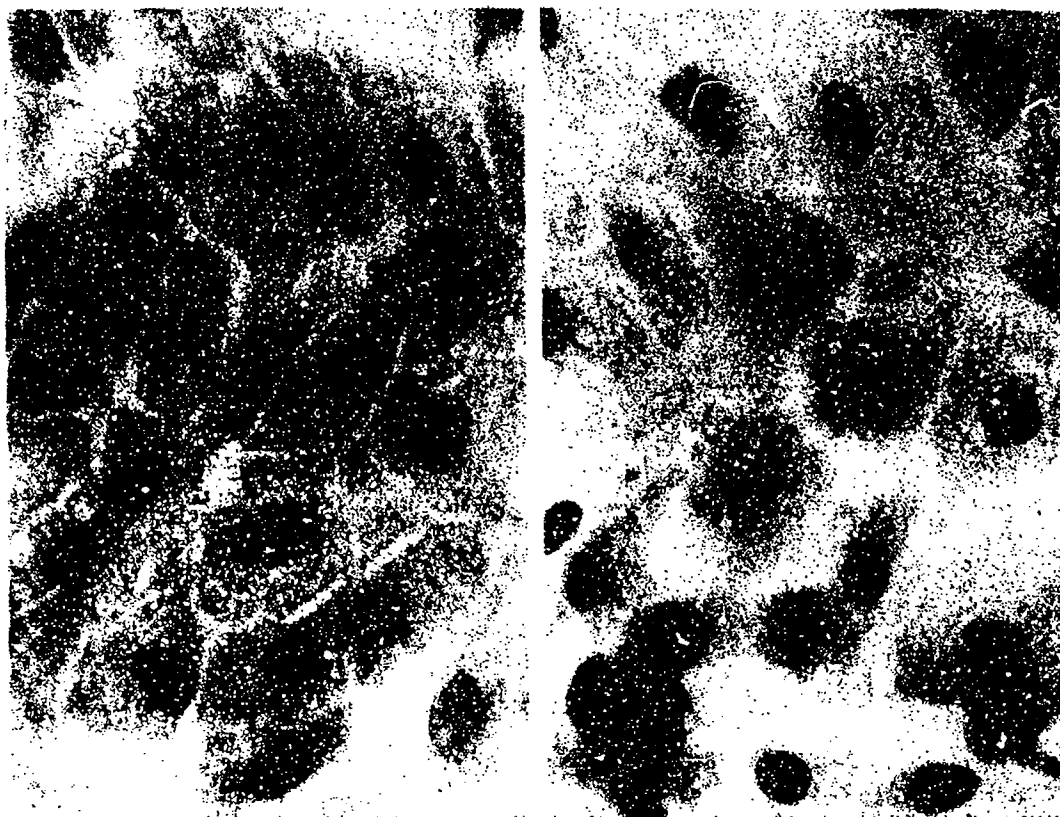


FIG. 2. — Effets de CBDZ sur explant primaire de rein de porc.

A gauche : culture témoin de 16 heures. Mitoses : 21/1.000. Pcnoses : 14/1.000.

A droite : culture libre de 12 heures puis CBDZ 2 mcg/ml pendant 4 heures. Mitoses : 89/1.000. Pcnoses : 40/1.000.

TABLEAU IV. — Effets sur l'évolution du carcinome d'Ehrlich de la souris Swiss.

Composés	Dose orale quotidienne	Nombre de Souris	T ₄ /P ₀	T ₈ /P ₀	Survie moyenne
témoin	0 mg/kg	94	24,4 %	42,2 %	13,44 j
BCM	100 + 100	15	24,4	53,9	13,38
BCM(NH)	100 + 100	14	25,6	51,0	11,63
PBDZ	100 + 100	15	10,4	15,9	10,57
	100	15	16,6	30,3	15,10
	75 + 75	15	5,0	25,4	17,92 (*)
OBDZ	200	15	27,6	43,4	13,43
OBDZ (**)	100 + 100	14	11,1 (*)		11,71
MBDZ	50 + 50	15	13,2		8,43
	30 + 30	14	15,9	28,6	18,14 (*)
FBDZ	200	15	25,6	43,9	13,82
OFDZ	100 + 100	15	26,9	44,9	12,71
	100	15	23,9	40,4	14,93
CyBDZ	50 + 50	14	17,8 (*)		10,39
DD-73	100 + 100	15	22,2	46,3	13,80
CBDZ	100 + 100	14	18,9		7,99
	100	22	18,8	30,3	11,58
	50 + 50	14	14,5	26,6	16,55 (*)
BZ.NH.Ac	75 + 75	14	20,9	38,3	12,62

(*) Significativement différent (test F) du témoin au risque de 5 p. 100.

(**) Administration sous-cutanée.

NH₂). En revanche, CO à lui seul n'est pas responsable de l'activité embryotoxique puisque les amides sont inactives (voir : BZ.NH.Ac, TBDZ.NH.Ac et TBDZ.NH.Bz). La substitution en N(3) est défavorable (comparer : BCM et BNM, CBDZ et CBDZ-NH₂). Ces règles se vérifient donc aussi bien en benzimidazolyl-5 qu'en benzimidazolyl-2. A l'intérieur de la série des N-benzimidazolyl-2, carbamates, l'embryotoxicité des divers représentants est extrêmement variable. La substitution en C(5) n'est pas indispensable puisque BCM est actif. En revanche, selon la nature du substituant, on passe de molécules très actives à des composés atoxiques dans les mêmes conditions expérimentales. Ce fait est d'autant plus frappant que les restes substituants peuvent être chimiquement très voisins (comparer : MBDZ et FBDZ, PBDZ et OBDZ).

Du point de vue antimitotique, on constate que pour la plupart des composés considérés, la corrélation entre activité *in vivo* et *in vitro* est élevée puisque les molécules très cytostatiques tant sur leucocytes que sur cellules KB (PBDZ, MBDZ, CBDZ, CyBDZ) sont également carcinoépreinatrices sur la tumeur d'Ehrlich ; à l'opposé, OFDZ et DD-73 sont inactifs sur les deux types de modèles expérimentaux. Quelques discordances sont cependant à remarquer, les plus évidentes étant relatives à BCM et OBDZ, tous deux actifs *in vitro* mais totalement dépourvus d'activité sur le carcinome de la souris. L'éventuelle liaison entre activité embryotoxique et activité anticancéreuse est vérifiée dans la majorité des cas tant parmi les molécules inactives (FBDZ, DD-73, OBDZ, BZ.NH.Ac) que parmi les composés actifs (PBDZ, MBDZ, CyBDZ, CBDZ). Des considérations métaboliques permettraient sans doute, parmi les N-benzimidazolyl-carbamates, d'éclaircir certaines discordances imputables au changement d'espèce animale (BCM embryotoxique chez le rat mais non anticancéreux chez la souris) ou à la voie d'administration (OBDZ inactif sur le carcinome d'Ehrlich par voie orale, mais actif par voie sous-cutanée). Certains de ces composés sont également actifs sur la leucémie L-1210 de la souris DBA/2 et BDF-1 [8, 11], sur la leucémie spontanée de la souris AKR [13] ainsi que sur la leucémie P-388. PBDZ et CBDZ semblent, enfin, doués d'effets favorables sur la leucose lymphoïde spontanée du chien. Par ailleurs, un dérivé N-benzimidazolyl-2, carbamate ne figurant pas dans la liste ci-dessus et proche de MBDZ a été trouvé actif sur le mélanocarcinome B 16 et le carcinome pulmonaire de LEWIS de la souris [1]. La nature des effets cytotoxiques observés *in vitro* sur cultures cellulaires permet de penser que le mode d'action de ces composés sur la cellule animale est identique à celui de BCM sur *Aspergillus nidulans* [4]. Il consisterait en une désorganisation des microtubules du fuseau mitotique et serait donc comparable à celui de la colchicine et de la vinblastine.

Les conséquences de ces résultats expérimentaux sont de divers ordres. Du point de vue de l'hygiène des denrées alimentaires d'origine animale, la persistance de résidus dans les organes des animaux d'élevage traités par les anthelminthiques PBDZ, MBDZ et CBDZ fait actuellement l'objet d'une attention particulière de la part de la Sous-Commission des Médicaments Vétérinaires de la Pharmacopée Française. Celle-ci s'applique spécialement à la détermination du délai d'attente, désormais légal (J. O. du 30 mai 1975), fondé sur la connaissance précise du métabolisme de ces médicaments chez les animaux. D'autre part, nous avons déjà eu l'occasion [8] de préconiser l'inscription de CBDZ au Tableau C des substances vénéneuses, mesure anté-

rieurement prise pour PBDZ (*J. O.* du 27 décembre 1974) et qui pourrait être étendue à MBDZ. Du point de vue médical, PBDZ qui au sein de cette série chimique offre la meilleure activité au regard de sa tolérance, a fait l'objet d'essais cliniques chez l'Homme (Prof. Cl. LAPRAS, Hôpital Neurologique, Lyon). L'intérêt de cette molécule semble confirmé sur néoblastomes et glioblastomes lors d'administration orale, infiltration locale ou injection intrarachidienne [14]. Par ailleurs, ces faits évoquent de nouvelles structures antimitotiques. Une démarche analogue à la nôtre a inspiré à l'Etranger l'étude expérimentale et clinique des propriétés anticancéreuses du composé très voisin qu'est le N((thiényl-2,carbonyl)5,benzimidazolyl)2,carbamate de méthyle [1] initialement breveté en tant qu'anthelminthique [20].

La poursuite de ces investigations, orientées par la relation structure/activité, pourrait permettre de découvrir de nouvelles molécules d'intérêt thérapeutique.

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EXHIBIT 8



US005880144A

United States Patent [19]**Camden**[11] **Patent Number:** **5,880,144**[45] **Date of Patent:** **Mar. 9, 1999**

[54] **PHARMACEUTICAL COMPOSITION FOR INHIBITING THE GROWTH OF VIRUSES AND CANCERS COMPRISING THIABENDAZOLE**

[75] **Inventor:** **James Berger Camden**, West Chester, Ohio

[73] **Assignee:** **The Procter & Gamble Company**, Cincinnati, Ohio

[21] **Appl. No.:** **927,550**

[22] **Filed:** **Sep. 6, 1997**

Related U.S. Application Data

[62] Division of Ser. No. 771,193, Dec. 20, 1996, Pat. No. 5,767,138.

[51] **Int. Cl.⁶** **A61K 31/47**

[52] **U.S. Cl.** **514/397; 514/398**

[58] **Field of Search** **514/397, 398**

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Primary Examiner—Frederick Krass

Attorney, Agent, or Firm—Rose Ann Dabek; Jacobus C. Rasser

[57] **ABSTRACT**

A pharmaceutical composition that inhibits the growth of tumors and cancers in mammals and can be used to treat viral infections that comprises a fungicide is disclosed. The particular fungicide used is a benzimidazole derivative.

15 Claims, No Drawings

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PHARMACEUTICAL COMPOSITION FOR INHIBITING THE GROWTH OF VIRUSES AND CANCERS COMPRISING THIABENDAZOLE

This is a division of application Ser. No. 08/771,193,
filed on Dec. 20, 1996, now U.S. Pat. No. 5,767,138.

TECHNICAL FIELD

This invention is a pharmaceutical composition that inhibits the growth of cancers and tumors in mammals, particularly in human and warm blooded animals. It is also effective against viruses and can be used to treat viral infections. The composition contains a benzimidazole derivative.

BACKGROUND OF THE INVENTION

Cancers are the leading cause of death in animals and humans. The exact cause of cancer is not known, but links between certain activities such as smoking or exposure to carcinogens and the incidence of certain types of cancers and tumors has been shown by a number of researchers.

Many types of chemotherapeutic agents have been shown to be effective against cancers and tumor cells, but not all types of cancers and tumors respond to these agents. Unfortunately, many of these agents also destroy normal cells. The exact mechanism for the action of these chemotherapeutic agents are not always known.

Despite advances in the field of cancer treatment the leading therapies to date are surgery, radiation and chemotherapy. Chemotherapeutic approaches are said to fight cancers that are metastasized or ones that are particularly aggressive. Such cytotoxic or cytostatic agents work best on cancers with large growth factors, i.e., ones whose cells are rapidly dividing. To date, hormones, in particular estrogen, progesterone and testosterone, and some antibiotics produced by a variety of microbes, alkylating agents, and anti-metabolites form the bulk of therapies available to oncologists. Ideally cytotoxic agents that have specificity for cancer and tumor cells while not affecting normal cells would be extremely desirable. Unfortunately, none have been found and instead agents which target especially rapidly dividing cells (both tumor and normal) have been used.

Clearly, the development of materials that would target tumor cells due to some unique specificity for them would be a breakthrough. Alternatively, materials that were cytotoxic to tumor cells while exerting mild effects on normal cells would be desirable. Therefore, it is an object of this invention to provide a pharmaceutical composition that is effective in inhibiting the growth of tumors and cancers in mammals with mild or no effects on normal cells.

More specifically, it is an object of this invention to provide an anti-cancer composition comprising a pharmaceutical carrier and a benzimidazole derivative as defined herein along with a method for treating such cancers.

These compositions are also effective against viruses and can be used to treat viral infections. Therefore it is another object of this invention to provide a method of treating viral infections such as HIV, influenza and rhinoviruses.

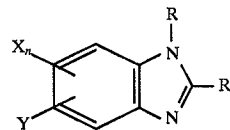
These and other objects will become evident from the following detailed description of this inventions.

SUMMARY OF THE INVENTION

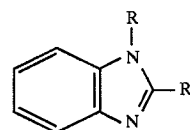
A pharmaceutical composition for treatment of mammals, and in particular, warm blooded animals and humans, comprising a pharmaceutical carrier and an effective amount

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anti-cancer compound selected from the group consisting of:



wherein X is hydrogen, halogen, alkyl of less than 7 carbon atoms or alkoxy of less than 7 carbon atoms; n is a positive integer of less than 4; Y is hydrogen, chlorine, nitro, methyl or ethyl; and R is hydrogen, or an alkyl group of from 1 to 8 carbon atoms and R₂ is 4-thiazolyl, NHCOOR₁ wherein R₁ is aliphatic hydrocarbon of less than 7 carbon atoms, and preferably an alkyl group of less than 7 carbon atoms is claimed. Preferably the compositions are:



wherein R is an alkyl of 1 through 8 carbon atoms and R₂ is selected from the group consisting of 4-thiazolyl, NHCOOR₁, wherein R₁ is methyl, ethyl or isopropyl and the non-toxic, pharmaceutically acceptable acid addition salts with both organic and inorganic acids. The most preferred compounds are 2-(4-thiazolyl)benzimidazole, methyl-(butylcarbamoyl)-2-benzimidazolecarbamate and 2-methoxycarbonylamino-benzimidazole and those wherein Y is chloro.

These compositions can be used to inhibit the growth of cancers and other tumors in humans or animals by administration of an effective amount either orally, rectally, topically or parenterally, intravenously or by injection into the tumor. These compositions do not significantly affect healthy cells as, compared to adriamycin which has a detrimental effect on healthy cells.

DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

As used herein, the term "comprising" means various components can be conjointly employed in the pharmaceutical composition of this invention. Accordingly, the terms "consisting essentially of" and "consisting of" are embodied in the term comprising.

As used herein, a "pharmaceutically acceptable" component is one that is suitable for use with humans and/or animals without undue adverse side effects (such as toxicity, irritation, and allergic response) commensurate with a reasonable benefit/risk ratio.

As used herein, the term "safe and effective amount" refers to the quantity of a component which is sufficient to yield a desired therapeutic response without undue adverse side effects (such as toxicity, irritation, or allergic response) commensurate with a reasonable benefit/risk ratio when used in the manner of this invention. The specific "safe and effective amount" will, obviously, vary with such factors as the particular condition being treated, the physical condition of the patient, the type of mammal being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the compounds or its derivatives.

As used herein, a "pharmaceutical addition salts" is salt of the anti-cancer compound with an organic or inorganic acid. These preferred acid addition salts are chlorides, bromides, sulfates, nitrates, phosphates, sulfonates, formates, tartrates, maleates, malates, citrates, benzoates, salicylates, ascorbates, and the like.

As used herein, a "pharmaceutical carrier" is a pharmaceutically acceptable solvent, suspending agent or vehicle for delivering the anti-cancer agent to the animal or human. The carrier may be liquid or solid and is selected with the planned manner of administration in mind.

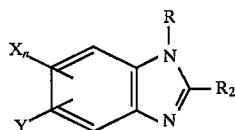
As used herein, "cancer" refers, to all types of cancers or neoplasm or malignant tumors found in mammals.

As used herein, the "anticancer compounds" are the benzimidazoles, and their salts. The exact benzimidazoles are described in detail below. The preferred materials are the products sold under the names "thiabendazole®", "benomyl®" and "carbendazim®" by BASF and Hoechst, DuPont and MSD-AgVet.

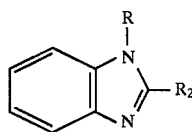
As used herein "viruses" includes viruses which infect animals or mammals, including humans. Viruses includes HIV, influenza, polio viruses, herpes, rhinoviruses, and the like.

B. The Anti-Cancer Compounds

The anti-cancer compounds are benzimidazole derivatives which are known for their antifungal activities. They are systemic fungicides used to prevent and eradicate fungi. The compounds have the following structure:



wherein X is hydrogen, halogen, alkyl of less than 7 carbon atoms or alkoxy of less than 7 carbon atoms; n is a positive integer of less than 4; Y is hydrogen, chlorine, nitro, methyl or ethyl; and R is hydrogen or an alkyl group having from 1 to 8 carbons, and R₂ is 4-thiazolyl, NHCOOR₁ wherein R₁ is aliphatic hydrocarbon of less than 7 carbon atoms, and preferably and alkyl group of less than 7 carbon atoms. Preferably the compositions are:



wherein R is an alkyl of 1 through 8 carbon atoms and R₂ is selected from the group consisting of 4-thiazolyl, NHCOOR₁, wherein R₁ is methyl, ethyl or isopropyl and the non-toxic, pharmaceutically acceptable acid addition salts with both organic and inorganic acids.

The most preferred compounds are 2-(4-thiazolyl) benzimidazole, methyl -(butylcarbamoyl)-2-benzimidazolecarbamate and 2-methoxycarbonylamino-benzimidazole and the compounds wherein Y is chloro and X is hydrogen.

These compounds are prepared according to the method described in U.S. Pat. No. 3,738,995 issued to Adams et al, Jun. 12, 1973. The thiazolyl derivatives are prepared according to the method described in Brown et al., *J. Am. Chem. Soc.*, 83 1764 (1961) and Grenda et al., *J. Org. Chem.*, 30, 259 (1965).

It is believed that fungicides, particularly systemic fungicides, have the capability of reducing tumors or decreasing their growth significantly. Systemic fungicides have the ability to migrate through the plant or animal body. While this is a positive attribute, it is not an essential requirement of the effective compounds for treating viral infections, cancers or tumors.

C. Dosage

Any suitable dosage may be given in the method of the invention. The type of compound and the carrier and the amount will vary widely depending on the species of the warm blooded animal or human, body weight, and tumor being treated. Generally a dosage of between about 2 milligrams (mg) per kilogram (kg) of body weight and about 400 mg per kg of body weight is suitable. Preferably from 15 mg to about 150 mg/kg of body weight is used. Generally, the dosage in man is lower than for small warm blooded mammals such as mice. A dosage unit may comprise a single compound or mixtures thereof with other compounds or other cancer inhibiting compounds. The dosage unit can also comprise diluents, extenders, carriers and the like. The unit may be in solid or gel form such as pills, tablets, capsules and the like or in liquid form suitable for oral, rectal, topical, intravenous injection or parenteral administration or injection into or around the tumor.

D. Dosage Delivery Forms

The anti-cancer compounds are typically mixed with a pharmaceutically acceptable carrier. This carrier can be a solid or liquid and the type is generally chosen based on the type of administration being used. The active agent can be coadministered in the form of a tablet or capsule, as an agglomerated powder or in a liquid form. Examples of suitable solid carriers include lactose, sucrose, gelatin and agar. Capsule or tablets can be easily formulated and can be made easy to swallow or chew; other solid forms include granules, and bulk powders. Tablets may contain suitable binders, lubricants, diluents, disintegrating agents, coloring agents, flavoring agents, flow-inducing agents, and melting agents. Examples of suitable liquid dosage forms include solutions or suspensions in water, pharmaceutically acceptable fats and oils, alcohols or other organic solvents, including esters, emulsions, syrups or elixirs, suspensions, solutions and/or suspensions reconstituted from non-effervescent granules and effervescent preparations reconstituted from effervescent granules. Such liquid dosage forms may contain, for example, suitable solvents, preservatives, emulsifying agents, suspending agents, diluents, sweeteners, thickeners, and melting agents. Oral dosage forms optionally contain flavorants and coloring agents. Parenteral and intravenous forms would also include minerals and other materials to make them compatible with the type of injection or delivery system chosen.

Specific examples of pharmaceutical acceptable carriers and excipients that may be used to formulate oral dosage forms of the present invention are described in U.S. Pat. No. 3,903,297 to Robert, issued Sep. 2, 1975. Techniques and compositions for making dosage forms useful in the present invention are described in the following references: 7 *Modern Pharmaceutics*, Chapters 9 and 10 (Banker & Rhodes, Editors, 1979); Lieberman et al., *Pharmaceutical Dosage Forms: Tablets* (1981); and Ansel, *Introduction to Pharmaceutical Dosage Forms 2nd Edition* (1976).

E. Method of Treatment

The method of treatment can be any suitable method which is effective in the treatment of the particular virus or

tumor type that is being treated. Treatment may be oral, rectal, topical, parenteral or intravenous administration or by injection into the tumor and the like. The method of applying an effective amount also varies depending on the tumor being treated. It is believed that parenteral treatment by intravenous, subcutaneous, or intramuscular application of the benzimidazole compounds, formulated with an appropriate carrier, additional cancer inhibiting compound or compounds or diluent to facilitate application will be the preferred method of administering the compounds to warm blooded animals.

The method of treating viral infections may also be by oral, rectal, topical, parenteral or intravenous administration. The actual time and dosage will depend on the virus being treated and the desired blood levels.

The following examples are illustrative and are not meant to be limiting to the invention.

Colon, Breast and Lung Tumor Cells Test

The following cell culture tests were performed to test the toxicity of the benzimidazole compounds on colon, breast and lung human tumor cells. The viability of the cells were tested by looking at MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction. MTT assay is a well known measure of cell viability.

The colon tumor cells (HT29 from American Type Culture Collection (ATCC)) and the breast cells (MX1 from cell lines from ATCC) were cultured in Eagle's Minimal Essential Medium with 10% fetal bovine serum. The lung tumor cells (A549 from ATCC cell lines) were cultured in Ham's F12 medium with 10% fetal bovine serum.

The tumor cells were passaged and seeded into culture flasks at the desired cell densities. The culture medium was decanted and the cell sheets were washed twice with phosphate buffered saline (PBS). The cells were trypsinized and triturated prior to seeding the flasks. Unless otherwise indicated the cultures were incubated at $37^{\circ}\pm 1^{\circ}$ C. in a humidified atmosphere of $5\pm 1\%$ carbon dioxide in air. The cultures were incubated until they were 50-80% confluent.

The cells were subcultured when the flasks were subconfluent. The medium was aspirated from the flasks and the cell sheets rinsed twice with PBS. Next, the Trypsin Solution was added to each flask to cover the cell sheet. The Trypsin Solution was removed after 30-60 seconds and the flasks were incubated at room temperature for two to six minutes. When 90% of the cells became dislodged, growth medium was added. The cells were removed by trituration and transferred to a sterile centrifuge tube. The concentration of cells in the suspension was determined, and an appropriate dilution was made to obtain a density of 5000 cells/ml. The cells were subcultured into the designated wells of the 96-well bioassay plates (200 microliter cell suspension per well). PBS was added to all the remaining wells to maintain humidity. The plates were then incubated overnight before test article treatment.

Each dose of test article was tested by treating quadruplicate wells of cultures with 100 microliter of each dilution. Those wells designated as solvent controls received an additional 100 microliter of methanol control; negative controls wells received an additional 100 microliters of treatment medium. PBS was added to the remaining wells not treated with test article or medium. The plates were then incubated for approximately 5 days.

At the end of the 5 day incubation, each dose group was examined microscopically to assess toxicity. A 0.5 mg/ml dilution of MTT was made in treatment medium, and the

dilution was filtered through a 0, 45 micrometer filter to remove undissolved crystals. The medium was decanted from the wells of the bioassay plates. Immediately thereafter, 2000 microliter of the filtered MTT solution was added to all test wells except for the two untreated blank test wells. The two blank wells received 200 microliters of treatment medium. The plates were returned to the incubator for about 3 hours. After incubation, the MTT containing medium was decanted. Excess medium was added to each well and the plates were shaken at room temperature for about 2 hours.

The absorbance at 550 nm (OD_{550}) of each well was measured with a Molecular Devices (Menlo Park, Calif.) VMax plate reader.

The mean OD_{550} of the solvent control wells and that of each test article dilution, and that of each of the blank wells and the positive control were calculated. The mean OD_{550} of the blank wells was subtracted from the mean of the solvent control wells, and test article wells, respectively to give the corresponding mean OD_{550} .

$$\% \text{ of Control} = \frac{\text{corrected mean } OD_{550} \text{ of Test Article Dilution}}{\text{corrected mean of } OD_{550} \text{ of Solvent Control}} \times 100$$

Dose response curves were prepared as semi-log plots with % of control on the ordinate (linear) and the test article concentration on the abscissa (logarithmic). The EC_{50} was interpolated from the plots for each test article.

For the test articles administered in methanol, separate responses were prepared to correct for the methanol data.

Adriamycin was used as a positive control. In all cases, it was more toxic than any of the test materials by one or two logs. Adriamycin is one of the more potent agents in current use and one with significant side effects. The peak plasma concentration of other, quite effective chemotherapeutic agents may be 10 to 50 times higher than that of Adriamycin.

The EC_{50} is the concentration at which one half of the cells are killed.

TABLE 1

Test Material	EC-50 Result (ppm)					
	HT29	HT29	MX1	MX1	A549	A549
Adriamycin	0.03	0.006	0.02	0.001	0.03	0.009
benomyl	0.742	0.747	1.42	2.42	0.980	1.02
carbendazim	0.621	0.662	0.829	0.856	0.856	0.836

In normal healthy cells, the following results were obtained. As is evident, the benomyl and carbendazim were much less toxic to normal healthy cells than adriamycin.

TABLE 2

Test Material	EC-50					
	Broncheal Cells		Keratinocyte Cells		Fibroblasts	
Benomyl	0.728	0.682	3.26	2.4	3.24	2.81
Carbendazim	0.320	0.506	0.752	0.822	1.52	1.42
Adriamycin	0.015	0.0020	0.0035	0.0093	0.065	0.10

In a related study using lung tumor cells (A-549) breast tumor cells (MCF-7) and colon tumor cells (HT-29), thiabendazol, a systemic fungicide, effectively killed these cells. Table 3 summarizes the results

TABLE 3

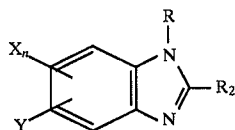
Concentration (ppm)	Optical Density		
	A-549	MCF-7	HT-29
0-Control	0.600	0.245	0.398
173	0.007	0.007	0.005
35	0.411	0.025	0.011
17.3	0.851	0.258	0.204
3.46	1.12	0.466	0.713
0.87	1.32	0.507	0.852

These experiments show that these compositions are effective in killing tumor cells.

These same systemic fungicides are effective against viruses including HIV, influenza, rhinoviruses and herpes viruses. The fungicides can be used alone or in combination with other fungicides.

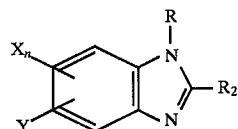
What is claimed is:

1. A method of treating viral infections in mammals comprising administering to a mammal in need thereof a safe and effective amount of a benzimidazole having the formula:



wherein X is hydrogen, halogen, alkyl of less than 7 carbon atoms or alkoxy of less than 7 carbon atoms; n is a positive integer of less than 4; Y is hydrogen, chlorine, nitro, methyl or ethyl; and R is hydrogen or an alkyl group having from 1 to 8 carbon atoms, and R₂ is 4-thiazolyl or the pharmaceutically acceptable organic or inorganic addition salts thereof.

2. A method of treating viral infections in mammals according to claim 1 by administering a safe and effective amount of a benzimidazole having the formula:

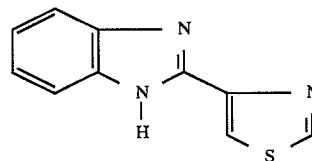


wherein X is hydrogen or halogen; n is 3; Y is hydrogen, and R is hydrogen or an alkyl group having from 1 to 8 carbon atoms, and R₂ is 4-thiazolyl.

3. A method according to claim 2 wherein the virus causing said viral infection is selected from the group consisting of HIV virus, influenza and rhinoviruses.

4. A method according to claim 1 wherein Y is chloro and wherein said benzimidazole is present in a composition comprising a pharmaceutical carrier.

5. A method of inhibiting HIV infections in mammals comprising administering to a patient in need thereof a safe and effective amount of a benzimidazole having the formula:



or the pharmaceutically acceptable acid addition salts thereof.

6. A method according to claim 5 wherein said pharmaceutically acceptable acid addition salts are selected from the group consisting of chlorides, bromides, sulfates, nitrates, phosphates, sulfonates, formates, tartrates, maleates, malates, citrates, benzoates, salicylates, ascorbates and mixtures thereof.

7. A method according to claim 5 wherein said HIV infection is chronic HIV.

8. A method according to claim 7 wherein from about 2 mg/kg body weight to about 400 mg/kg of said benzimidazole is administered.

9. A method according to claim 4 wherein said benzimidazole is administered orally or enterically, intravenously, peritoneally, or by injection.

10. A method according to claim 4 wherein said benzimidazole is administered in a solid form.

11. A method according to claim 10 wherein said solid form includes a carrier selected from the group consisting of lactose, sucrose, gelatin and agar.

12. A method according to claim 11 wherein from about 15 mg/kg body weight to about 150 mg/kg of said benzimidazole is administered.

13. A method according to claim 4 wherein said benzimidazole is administered in a liquid form.

14. A method according to claim 13 wherein said liquid dosage form is selected from the group consisting of aqueous solutions, alcohol solutions, emulsions, suspensions, and suspensions reconstituted from non-effervescent and effervescent preparations and suspensions in pharmaceutically acceptable fats or oils.

15. A method according to claim 13 wherein said liquid dosage form contains an additive which is selected from the group consisting of suspending agents, diluents, sweeteners, flavorants, colorants, preservatives, emulsifying agents and coloring agents, and mixtures thereof.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,880,144
DATED : March 9, 1999
INVENTOR(S) : James B. Camden

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page.

Under **Related U.S. Application Data**, following "5,767,138" insert --, which is a division of Ser. No. 08/420,914, Apr. 12, 1995, abandoned --.

Claim 1.

Line 10 (col. 7, line 34), delete "4-thiazolyl or" and insert in lieu thereof -- 4-thiazolyl; or --.

Claim 9.

Line 2 (col. 8, line 28), delete "orally or enterically," and insert in lieu thereof -- orally, enterically, --.

Signed and Sealed this

Twenty-seventh Day of November, 2001

Attest:

Nicholas P. Godici

Attesting Officer

NICHOLAS P. GODICI
Acting Director of the United States Patent and Trademark Office

EXHIBIT 9

Computer-Assisted Structure-Anticancer Activity Correlations of Carbamates and Thiocarbamates

MOHAMED NASR*, KENNETH D. PAULL[‡], AND V. L. NARAYANAN^{*x}

Received February 2, 1985, from *Starks C. P., Rockville, MD 20852 and the [‡]Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20205. Accepted for publication April 24, 1985.

Abstract □ With the aid of the computer, ~8000 compounds that incorporate a carbamate or thiocarbamate moiety, which have been tested as potential anticancer agents at the National Cancer Institute (NCI), were classified and their structure-activity correlations against the *in vivo* P-388 and L-1210 leukemias were evaluated. Aromatic carbamates and thiocarbamates have shown good activity against P-388 and poor activity against L-1210. The majority of active compounds in this series of aromatic carbamates possess a 2- or 4-heteroatom-substituted phenyl attached to the carbamate oxygen atom or the thiocarbamate sulfur atom with the carbamate nitrogen atom as NHMe. The *N*-phenyl carbamates were much less active against P-388 than the phenyl carbamates; only bis-*N*-phenyl carbamates with a methylene bridge between the two phenyl groups showed good activity against both P-388 and L-1210 leukemias. Except for the mycophenolic acid carbamates, the fused phenyl carbamates showed poor activity against both P-388 and L-1210 leukemias. Certain nitrogen-heterocyclic carbamates and carbamates with heteroatom substituents have been selected by the NCI for development toward clinical trials. The nature of the heterocyclic carrier and the position of attachment to the carbamate moiety have a major role on the mode of action of the antitumor activity of these compounds.

sent an important class of potential anticancer compounds, some of which have been used clinically and some which are under development toward clinical trials.⁶ Because of the diverse application of carbamates, both as insecticides⁷ and as therapeutic agents,^{8,9} a great deal of work has been done on the synthesis,^{10,11} metabolism, and toxicology of carbamates and thiocarbamates.¹⁰⁻¹⁷ Over 8000 compounds incorporating a carbamate or thiocarbamate moiety have been evaluated as potential anticancer agents at the NCI. In this article we report the structural types and the *in vivo* anticancer activity correlations of aromatic, aliphatic, and heterocyclic carbamates and their thio-isoesters against both intra-peritoneally implanted murine P-388 lymphocytic leukemia and L-1210 lymphoid leukemia.¹⁸

Experimental Section

The study takes advantage of the computer's ability to search substructures according to precise definitions and to manipulate these substructures utilizing Boolean logic. The method of analysis has been described in detail in previous articles.^{3,4}

Results and Discussion

The results of the analysis are summarized in Tables I-IX. Each table represents a basic structural type and shows the effect of structural modification on the antitumor activity. Each table shows the number of compounds of a particular

As a part of our strategy for acquiring novel compounds for anticancer evaluation, we have undertaken structure-antitumor activity correlation studies of different classes¹ of compounds that have been tested at the National Cancer Institute (NCI).²⁻⁵ Carbamates and thiocarbamates repre-

Table I—Phenyl Carbamates 1

No.	Substituents ^a	P-388		L-1210		Examples	
		No. Tested	Percent Active	No. Tested	Percent Active	NSC ^b	Structure
1a	R = Any, no fusion allowed on benzene ring	956	10	1491	0.4		
1b	R = H ₂	5	20	7	0	66509	R = H ₂
1c	R = HMe	340	15	393	0.6	11846	R = HMe, 2,6-Me, 4-OCONHMe
1d	R = HEt	8	25	14	0	194892	R = HEt, 2-Cl, 4-SO ₂ F
1e	R = Dialkyl	55	2	115	0	194867	R = Me ₂ , 2-Cl, 4-SO ₂ F
1f	NR = N(NO)CH ₂ CH ₂ X	14	76	2	50	330782	NR = NCH ₂ CH ₂ Cl, 2,6-Cl NO
1g	R = HT	55	7	38	0	144056	R = HSO ₂ -
1h	2,4-HT	138	29	207	0.6	18115	R = HMe, 2,4,5-Cl
1i	2-X, 4-S	27	56	49	0	39642	R = HMe, 2-Cl, 4-SPhOCONHMe
1j	3-S	3	100	8	0	268919	R = HMe, 3-S(CH ₂) ₁₁ Me, 4-OCONHMe, 5,6-OMe

^a HT = Any atom except C or H; X = halogen. ^b The NSC identify each compound in the NCI file, and upon request antitumor data and other information can be retrieved for each NSC number.

Table II—N-Phenyl Carbamates 2

No.	Substituents	P-388		L-1210		Examples	
		No. Tested	Percent Active	No. Tested	Percent Active	NSC	Structure
2a	R = Any, except phenyl—no fusion allowed	1391	4	1079	1		
2b	R = CC≡C	174	6	29	0	29175	R = CH ₂ C≡CCH ₂ Cl, 4-Cl R = CH ₂ C≡CCH ₂ Cl, 3-Cl
2c	R = CC≡CCX	82	9	2	0	29168	
2d	R = Nonring N	256	3	43	1	405907	
2e	R = Ring N	58	6	3	0	305189	
2f	4-Y--Z	21	13	16	17	25182	R = Et, 4-SO ₂ -
2g	4-CH ₂ --NHCOOR					215914	R = Me, 4-CH ₂ -

Table III—Fused Phenyl Carbamates 3

No.	Substructure	P-388		L-1210		Examples	
		No. Tested	Percent Active	No. Tested	Percent Active	NSC	Structure
3a		134	5	192	7	43669	
3b		3	100	34	38	191323	
3c		25	0	41	1	19715	
3d		133	3	107	1	30504	

type that have been tested against P-388 and/or L-1210 leukemias and the percentage of active compounds.^{3,4} For each type an example representing the active compounds is given, except in cases where no active compounds were found.

The information generated by this method is indicative rather than definitive. The presence of relatively many active compounds within an analyzed group is considered a reasonable basis for additional, more thorough studies on the group. For a variety of good reasons, a high percentage of activity cannot be considered proof that the subject substructure is required for the antitumor activity or even relevant to it. On the other hand, if few active compounds are found among a relatively large group having a given substructure, it is safe to assume that the particular substructure is not particularly relevant to that type of anticancer activity.

In Table I, the activity of phenyl carbamates against the P-388 and L-1210 leukemias are described. Phenyl carbamates of type 1a showed ~10% overall activity against P-388 and poor activity against L-1210. The P-388 activity varies considerably with substituents on the carbamate nitrogen atom and on the benzene ring. Compounds with heteroatom substituents at the 2- and 4-position of the benzene ring, 1h, and the carbamate nitrogen atom as HN-alkyl, 1c and 1d, showed good activity against P-388. Compounds with a sulfur atom at the 4-position and halogen at the 2-position, 1i showed excellent activity. The three compounds with a sulfur substituent at the 3-position, 1j, that were tested were all active. Phenyl *N,N*-dialkylated carbamates 1e are the least active of all the different classes. However, phenyl *N*-hydroxyalkyl carbamates, a common active metabolite of the carbamate insecticides,¹³ have not been evaluated as antitumor agents.

Table IV—Thiocarbamates 4

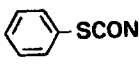
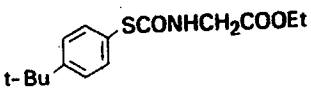
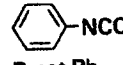
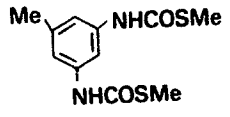

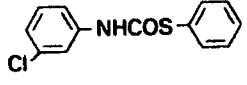
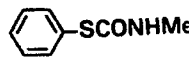
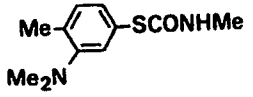
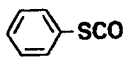
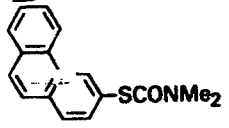
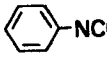
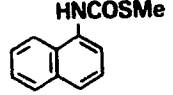
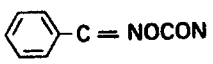
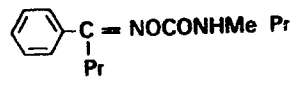
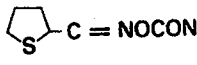
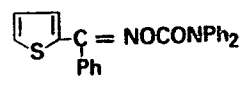
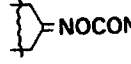

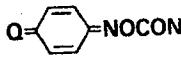
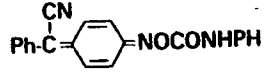
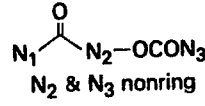

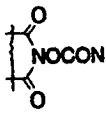
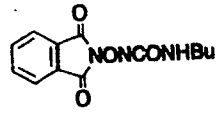
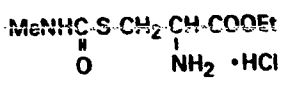
No.	Substructure	P-388		L-1210		Examples	
		No. Tested	Percent Active	No. Tested	Percent Active	NSC	Structure
4a	 No Fusion Allowed	90	31	163	1	241633	
4b	 R not Ph No fusion allowed	257	5	72	0	38377	
4c		24	24	53	0	30761	
4d		23	74	23	1	128137	
4e	 Only fused systems	2	33	4	0	171701	
4f	 Only fused systems	5	0	2	0	59319	

Table V—Carbamate Oximes, Ureas, and Imides 5

No.	Substructure ^a	P-388		L-1210		Examples	
		No. Tested	Percent Active	No. Tested	Percent Active	NSC	Structure
5a		196	2	52	1	107731	
5b		30	7	17	2	115155	
5c		104	5	32	0	2492	
5d		31	15	7	0	405807	
5e	 N ₂ & N ₃ nonring	46	34	5	0	253272	
5f		16	14	2	0	305188	
5g						303861	

^aQ = any substituent.

Table VI—Carbamates with the Nitrogen Atom as a Part of a Heterocyclic Ring 6

No.	Substructure	P-388		L-1210		Examples	
		No. Tested	Percent Active	No. Tested	Percent Active	NSC	Structure
6a		503	2	291	1	22151	
6b		122	6	27	0	165897	
6c		10	20	4	0	142799	
6d		21	10	4	0	59322	
6e		14	13	3	0	148791	
6f		12	0	15	0	150068	


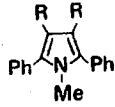

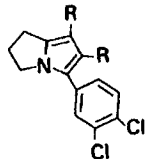



Table VII—Heterocyclic Carbamates 7

No.	R	Y	P-388		L-1210		Examples	
			No. Tested	Percent Active	No. Tested	Percent Active	NSC	Structure
7a	Ph	NH	21	79	6	67	330770	
7b	Any	=N	1	0	3	0	337238	
7c	Any	O	10	30	1	100	344270	
7d	Any	S	5	0	0		302066	

Table VIII—Benzimidazole Carbamates 8

No.	R	Y	P-388		L-1210		Examples	
			No. Tested	Percent Active	No. Tested	Percent Active	NSC	Structure
8a	Any	N	107	22	20	21	184849	
8b	Me	NH	33	51	13	33	335307	
8c	Any	S	50	3	4	0	327383	
8d	Any	O	—	—	—	—		

Table IX—Pyrroles and Fused Pyrroles 9

No.	Substructure	P-388		L-1210		NSC	Examples
		No. Tested	Percent Active	No. Tested	Percent Active		Structure
9a	 $R = \text{CH}_2\text{OCON}$ No fusion allowed	34	100	4	42	301486	 $R = \text{CH}_2\text{OCONHMe}$
9b	 $R = \text{CH}_2\text{OCON}$	39	92	12	42	278214	 $R = \text{CH}_2\text{OCONHCH(Me)}_2$
9c		3	0	0		329303	
9d		1	0	0			

The only class that showed good activity against both the P-388 and L-1210 leukemias are 2-chloroethyl nitrosocarbamates 1f.¹⁰

Table II describes the activity of *N*-phenyl carbamates. In general, these are much less active than phenyl carbamates. Compounds of type 2f showed good activity against both P-388 and L-1210 leukemias. The active subset of 2f is type 2g, and these have shown good activity against a variety of other tumor test systems, e.g., B16 melanoma. Acetylenic carbamates 2c showed moderate activity against P-388. Contrary to phenyl carbamates, the nature of substitution on the phenyl group in *N*-phenyl carbamates did not influence the antitumor activity.

Table III represents the antitumor evaluation of compounds with the phenyl ring fused with other systems. Naphthalene carbamates 3c are inactive against both P-388 and L-1210. Fused *N*-phenyl carbamates 3d showed low activity against P-388 and L-1210. Mycophenolic acid carbamates^{19,20} 3b are the only type that showed excellent activity against L-1210, which in general is much more resistant than P-388.

Phenyl thiocarbamates, which show good activity against P-388 and poor activity against L-1210, are described in Table IV. The presence of an NHMe group, as in 4d, confers the best activity. Phenyl thiocarbamates 4a and 4c showed greater activity against P-388 than *N*-phenyl thiocarbamates 4b. In general, phenyl thiocarbamates showed better activity against P-388 than phenyl carbamates.

Table V describes carbamate oximes, ureas, and cyclic nitrogen carbamates. Substructure types 5d–5f have shown good activity against P-388 and are inactive against L-1210. Aromatic carbamate oximes 5a are less active against P-388 than the cyclic oximes 5c and the heterocyclic oximes 5b. It should be mentioned that while carbamates have shown poor activity against L-1210 and a moderate level of activity against P-388 some have shown excellent activity against the mammary xenograft tumor test system 3MBG5,¹⁸ e.g., the *L*-cysteine derivative²¹ 5g and caracemide^{22,23} 5e (5e is under clinical trials at NCI). It should be noted that among the 16 mammary-active synthetic carbamates (excluding mitomycins and other natural products), 6 compounds are *N*-

methylcarbamate derivatives. *N*-Methylformamide^{24,25} has also shown excellent activity against the mammary xenograft.^{6,18} There may be a relationship between the mammary activity and the presence of the C(O)NHMe grouping.

Carbamates and thiocarbamates with the carbamate nitrogen atom as a part of a ring system are described in Table VI. In general, the carbamates of this class showed poor activity while the thiocarbamates possess moderate activity against P-388. Triazole carbamates 6c showed good activity against P-388 and were inactive against L-1210. Piperidine thiocarbamates 6d and 2-cyano-*N*-thiocarbamates 6e showed moderate activity against P-388 lymphocytic leukemia.

Heterocyclic carbamates (Table VII) show high activity against both P-388 and L-1210. 1,2-Dihydropyrido[3,4-*b*]pyrazines 7a represent the main type tested and are the most active. These compounds have been reported to exert their antitumor activity as tubulin binders.^{26–29} Oxidation of 7a to the corresponding heteroaromatic system 7b abolishes the activity in both P-388 and L-1210. Compounds of structure 7c, Y = O, still retain activity but with a lower percentage of activity against P-388 than 7a, while those with Y = S, 7d, were devoid of any activity against P-388. It has been stated that the absence of antitumor activity of pyridothiazines and the higher doses required for activity of the pyridoxazine analogues of 1,2-dihydropyrido[4,3-*b*]pyrazines indicate that the 1-NH of the latter plays an important role in binding to tubulin.²⁹

Benzimidazole carbamates of type 8a, which have shown good activity against both P-388 and L-1210, are represented in Table VIII. Optimal activity was obtained with Y = NH and R = CH₃, 8b. When Y = S, 8c, the activity against P-388 decreased to 3%. No compounds with Y = O were tested in either the P-388 or L-1210 screens. The antitumor activity of these compounds may also be related to their tubulin binding ability.^{30,31}

Table IX represents an important group of pyrroles and fused pyrrole biscarbamates³² showing excellent antitumor activity. Some of these compounds have shown a broad spectrum of antitumor activity, including P-388, L-1210, mammary xenograft, and B16 melanoma. This antitumor activity can be explained as alkylation occurring through

facile O-alkyl cleavage of the carbamate moiety enhanced by participation of the ring nitrogen. The development of these compounds are hampered because of poor water solubility and stability. New approaches are needed to solve these problems in order to develop the compounds of this class to the clinical stage.

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Another Anniversary for the War on Cancer

GERALD B. DERMER

Gerald B. Dermer, a former cancer researcher, is the author of *The Immortal Cell: Why Cancer Research Fails*, published by Avery Publishing Group, Garden City Park, NY. He can be reached at 1235 N. Sunnyvale, No. 87, Mesa, AZ 85205. The views expressed here are the author's own and not necessarily those of Bio/Technology.

In 1993, 526,000 Americans died of cancer—about 1400 people a day—even though conquering cancer became a national goal in December, 1971, now more than 22 years ago. Though tens of thousands of skilled scientists have been waging war against cancer in their laboratories for a generation, spending billions of tax, charitable, and investor dollars, the dread disease remains a metaphor for anything evil in society that spreads. The human statistics should be an issue of the most profound concern to the cancer industry because they are the only true measure of its performance, and the statistics remain very grim.

Why don't we have a cancer cure by now? The answer, in my opinion, is basic and essentially simple: The cell lines in which cancer is usually studied are unsuitable for the job. They do not mimic conditions in the human body.

The cancer industry has ignored the limitations of its most important piece of material—its favorite model—because "Nobody likes to ask if a model is really correct..." (Francis Crick, *What Mad Pursuit*, 1988, Basic Books, New York, p. 161). More than 40 years ago, these long-term cell cultures began their careers as stand-ins for real cancer based only on investigator faith in their reliability. Because they are so convenient for experimentation and the methods of molecular biology, cell lines today have become the standard for determining what cancer should be like. The facts indicate, however, that petri dish cancer is really a poor representation of malignancy, with characteristics profoundly different from the human disease.

When a normal or malignant body cell survives a crisis period and adapts to immortal life in culture, it takes an evolutionary-type step that enables the new line to thrive in its artificial environment. This step transforms a cell from one that is stable and differentiated to one that is not. Yet normal or malignant cells *in vivo* are not like that. This means that cell lines are really a new life form on Earth, neither human nor animal. Evidence of the contradictions between life on the bottom of a lab dish and in the body has been in the scientific literature for more than 30 years, evidence that has been systematically ignored by the cancer establishment.

Studies of human and animal cancer have shown that only differentiating, aging cells in organs are susceptible to cancer. Data from undifferentiated, ageless "normal" cell lines—like 3T3 in which the pathways that are struck by cancer, those of develop-

ment and aging, are absent—cannot be relevant to cancer initiation in humans.

The widely disparate character of human tumor cell lines contributes greatly to chemotherapy's continued ineffectiveness against cancer. New drugs are selected for human trials because they kill tumor cell lines in the laboratory.

Surgical pathologists, the specialists who diagnose cancer, have long recognized that cancer cells are just misbehaving body cells. In other words, the immune system registers self when confronted by a malignant cell. This means that several decades of highly publicized, well-funded research on immunotherapy has produced only mice that are cured of their cell line tumors.

The standard approach of most cancer scientists to experimentation produces little of practical value because it is flawed. Typically, an observation is first made in culture, then the investigator turns to human cancer. If the observation is duplicated, papers are written about the significant find. But, if you look long and hard enough *in vivo* you will always find what you seek. For example, there may be a rare human tumor that is immunogenic, but this is just the exception that proves the rule. There are human tumors in which a proto-oncogene is mutated, but there are others of the same type in which it is not. What is significant in culture, for example immunotherapy's killing power or the transformation of 3T3 cells by a mutated proto-oncogene, simply does not have the same significance for cells *in vivo*. Instead of this approach, models that mimic the human body and the developmental pathways of human cells, both normal and malignant, should be first identified. Only then will truly significant observations be made.

How cancer is defined today depends on what courses have been taken, what books have been read, which journals have been studied, and what research training and practice have been followed. The result is confusion. An unnatural condition created in the laboratory is being mistaken for human cancer.

Every year, for as long as I can remember, cancer scientists and cancer physicians have met during the same week, under one roof, at overlapping conferences. They no longer will do so. This year, the American Association for Cancer Research and the American Association of Clinical Oncology begin meeting separately. The new meeting policy, initiated by the researchers, is as open an admission as one is likely to get from them that they really haven't been interested in the real world for a long time. ///

CULTURE OF ANIMAL CELLS

A Manual of
Basic Technique

R. IAN Freshney

Other areas of major interest include the study of cell interactions and intracellular control mechanisms in cell differentiation and development [Auerbach and Grobstein, 1958; Cox, 1974; Finbow and Pitts, 1981] and attempts to analyze nervous function [Bornstein and Murray, 1958; Minna et al., 1972]. Progress in neurological research has, however, not had the benefit of working with propagated cell lines as propagation of neurons has not so far been possible *in vitro* without resorting to the use of transformed cells (see Chapter 20).

Tissue culture technology has also been adopted into many routine applications in medicine and industry. Chromosomal analysis of cells derived from the womb by amniocentesis can reveal genetic disorders in the unborn child, viral infections may be assayed qualitatively and quantitatively on monolayers of appropriate host cells, and the toxic effects of pharmaceutical compounds and potential environmental pollutants can be measured in colony-forming assays.

Further developments in the application of tissue culture to medical problems may follow from the demonstration that cultures of epidermal cells form functionally differentiated sheets in culture [Green et al., 1979], and endothelial cells may form capillaries [Folkman and Haudenschild, 1980], suggesting possibilities in homografting and reconstructive surgery using an individual's own cells. The introduction of heterologous genetic material into mammalian cells [Willecke et al., 1979; Wigler et al., 1979], although somewhat overshadowed by current propagation in bacteria, may yet prove a desirable means for producing biologically significant compounds such as growth hormone and insulin. Similarly, the production of monoclonal antibodies [Kohler and Milstein, 1975] in hybrids between human plasma cells and human myeloma cells may prove a valuable technique for the production of specific antibodies.

It is clear that the study of cellular activity in tissue culture may have many advantages; but in summarizing these, below, considerable emphasis must also be placed on its limitations, in order to maintain some sense of perspective.

ADVANTAGES OF TISSUE CULTURE

Control of the Environment

The two major advantages, as implied above, are the control of the physicochemical environment (pH, temperature, osmotic pressure, O_2 , CO_2 tension),

which may be controlled very precisely, and the physiological conditions, which may be kept relatively constant but cannot always be defined. Most media still require supplementation with serum which is highly variable [Olmsted, 1967; Honn et al., 1975], and contains undefined elements such as hormones and other regulatory substances. Gradually, however, the functions of serum are being understood; and as a result, it is being replaced by defined constituents [Birch and Pirt, 1971; Ham and McKeehan, 1978; Barnes and Sato, 1980].

Characterization and Homogeneity of Sample

Tissue samples are invariably heterogeneous. Replicates even from one tissue vary in their constituent cell types. After one or two passages, cultured cell lines assume a homogeneous, or at least uniform, constitution as the cells are randomly mixed at each transfer and the selective pressure of the culture condition tends to produce a homogeneous culture of the most vigorous cell type. Hence, at each subculture each replicate sample will be identical, and the characteristics of the line may be perpetuated over several generations. Since experimental replicates are virtually identical, the need for statistical analysis of variance is seldom required.

Economy

Cultures may be exposed directly to a reagent at lower and defined concentration, and with direct access to the cell. Consequently, less is required than if injection *in vivo* where >90% is lost by excretion and distribution to tissues other than those under study.

DISADVANTAGES

Expertise

Culture techniques must be carried out under sterile aseptic conditions, because animal cells grow much less rapidly than many of the common contaminants such as bacteria, molds, and yeasts. Furthermore, like microorganisms, cells from multicellular animals do not exist in isolation, and consequently, are not able to sustain independent existence without the provision of a complex environment, simulating blood plasma and interstitial fluid. This implies a level of skill and understanding to appreciate the requirements of the system and to diagnose problems as they arise. Tissue culture should not be undertaken casually to run one or two experiments.

Quantity

A major limitation of cell culture is the expenditure of effort and materials that goes into the production of relatively little tissue. A realistic maximum per batch for most small laboratories (2 or 3 people doing tissue culture) might be 1–10 g of cells. With a little more effort and the facilities of a larger laboratory, 10–100 g is possible; above 100 g implies industrial pilot plant scale, beyond the reach of most laboratories, but not impossible if special facilities are provided.

The cost of producing cells in culture is about ten times that of using animal tissue. Consequently, if large amounts of tissue (> 10 g) are required, the reasons for providing them by tissue culture must be very compelling. For smaller amounts of tissue (≤ 10 g), the costs are more readily absorbed into routine expenditure; but it is always worth considering whether assays or preparative procedures can be scaled down. Semimicro- or micro-scale assays can often be quicker due to reduced manipulation times, volumes, centrifuge times, etc. and are often more readily automated (see under Microtitration, Chapter 19).

Instability

This is a major problem with many continuous cell lines resulting from their unstable aneuploid chromosomal constitution. Even with short-term cultures, although they may be genetically stable, the heterogeneity of the cell population, with regard to cell growth rate, can produce variability from one passage to the next. This will be dealt with in more detail in Chapters 12 and 18.

MAJOR DIFFERENCES *IN VITRO*

Many of the differences in cell behavior between cultured cells and their counterparts *in vivo* stem from the dissociation of cells from a three-dimensional geometry and their propagation on a two-dimensional substrate. Specific cell interactions characteristic of the histology of the tissue are lost, and, as the cells spread out, become mobile and, in many cases, start to proliferate, the growth fraction of the cell population increases. When a cell line forms it may represent only one or two cell types and many heterotypic interactions are lost.

The culture environment also lacks the several systemic components involved in homeostatic regulation *in vivo*, principally those of the nervous and endocrine systems. Without this control, cellular metabolism may

be more constant *in vitro* than *in vivo*, but may not be truly representative of the tissue from which the cells were derived. Recognition of this fact has led to the inclusion of a number of different hormones in culture media (see Chapter 9) and it seems likely that this trend will continue.

Energy metabolism *in vitro* occurs largely by glycolysis, and although the citric acid cycle is still functional it plays a lesser role.

It is not difficult to find many more differences between the environmental conditions of a cell *in vitro* and *in vivo* and this has often led to tissue culture being regarded in a rather skeptical light. Although the existence of such differences cannot be denied, it must be emphasized that many specialized functions are expressed in culture and as long as the limits of the model are appreciated, it can become a very valuable tool.

Origin of Cells

If differentiated properties are lost, for whatever reason, it is difficult to relate the cultured cells to functional cells in the tissue from which they were derived. Stable markers are required for characterization (see Chapter 15); and in addition, the culture conditions may need to be modified so that these markers are expressed (see next chapter).

DEFINITIONS

There are three main methods of initiating a culture [Schaeffer, 1979] (see Glossary and Fig. 1.2): (1) *Organ culture* implies that the architecture characteristic of the tissue *in vivo* is retained, at least in part, in the culture. Toward this end, the tissue is cultured at the liquid/gas interface (on a raft, grid, or gel) which favors retention of a spherical or three-dimensional shape. (2) In *primary explant culture* a fragment of tissue is placed at a glass (or plastic)/liquid interface where, following attachment, migration is promoted in the plane of the solid substrate. (3) *Cell culture* implies that the tissue or outgrowth from the primary explant is dispersed (mechanically or enzymatically) into a cell suspension which may then be cultured as an adherent monolayer on a solid substrate, or as a suspension in the culture medium.

Organ cultures, because of the retention of cell interactions as found in the tissue from which the culture was derived, tend to retain the differentiated properties of that tissue. They do not grow rapidly (cell proliferation is limited to the periphery of the explant and is restricted mainly to embryonic tissue) and hence ca

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Printed in the United States of America

Second Printing, April 1984
Third Printing, November 1984
Fourth Printing, November 1986

Library of Congress Cataloging in Publication Data

Freshney, R. Ian.
Culture of animal cells.

Bibliography: p.
Includes index.

1. Tissue culture. 2. Cell culture. I. Title.
QH585.F74 1983 591'.07'24 82-24960
ISBN 0-8451-0223-0

EXHIBIT 10

Multidrug Resistance Modulators and Doxorubicin Synergize to Elevate Ceramide Levels and Elicit Apoptosis in Drug-Resistant Cancer Cells

Anthony Lucci, M.D.¹

Tie-Yan Han, M.D.²

Yong-Yu Liu, M.D., Ph.D.²

Armando E. Giuliano, M.D.¹

Myles C. Cabot, Ph.D.²

¹ Department of Surgical Oncology, John Wayne Cancer Institute at Saint John's Health Center, Santa Monica, California.

² John Wayne Cancer Institute at Saint John's Health Center, Santa Monica, California.

Presented in part at the 33rd Annual Meeting of the American Society of Clinical Oncology, Denver, Colorado, May 17-20, 1997.

Supported in part by a fellowship training grant from the Society of Surgical Oncology (A.L.); the Breast Cancer Fund of the State of California through the Breast Cancer Research Program of the University of California (grant 0211 to M.C.C.); the John Wayne Cancer Institute Auxiliary; The Associates for Breast Cancer Studies (ABCs), Los Angeles; the Fashion Footwear Association of New York (FFANY) Shoes on Sale; and the National Institutes of Health, (R01 CA77632 to M.C.C.).

A. Lucci's present address is Department of Surgery, Baylor College of Medicine 6550 Fannin, Suite 1628, Houston, TX 77030.

Address for reprints: Myles C. Cabot, John Wayne Cancer Institute, at Saint John's Health Center, 2200 Santa Monica Blvd., Santa Monica, CA 90404.

Received October 15, 1998; revision received February 5, 1999; accepted February 26, 1999.

BACKGROUND. To provide insight for the development of more effective clinical agents, the authors attempted to elucidate the mechanisms of action of multidrug resistance (MDR) modulators. Previously, the authors found that MDR modulators blocked the conversion of ceramide to glucosylceramide in MDR cells, thereby enhancing cytotoxicity. Because ceramide is a critical component of the apoptosis signaling cascade, the current study examined the impact of therapy using agents that elicit ceramide formation combined with agents that block ceramide glycosylation.

METHODS. Doxorubicin-resistant human breast carcinoma cells (MCF-7-AdrR) were treated with either doxorubicin, tamoxifen, cyclosporine A, or the cyclosporine A analog SDZ PSC 833 (PSC 833) or with combinations thereof, and ceramide and glucosylceramide metabolisms were measured by cell radiolabeling. Cell viability was quantitated spectrophotometrically and apoptosis was evaluated analyzing DNA integrity by gel electrophoresis.

RESULTS. Whereas cyclosporine A blocked the generation of glucosylceramide in MCF-7-AdrR cells, a chemical cousin, PSC 833, elicited a 3-fold increase in glucosylceramide and a 5-fold increase in ceramide levels at 24 hours. The PSC 833 response was time-dependent (as early as 30 minutes) and dose-dependent (as low as 0.1 μ M). The appearance of ceramide foreran the generation of glucosylceramide. Sphingomyelin levels were not decreased in response to PSC 833; however, Fumonisin B₁, a ceramide synthase inhibitor, blocked PSC 833-induced ceramide generation. Adding tamoxifen, which blocks ceramide glycosylation, to the PSC 833 regimen boosted ceramide levels 11-fold over controls and caused DNA fragmentation. A 3-component regimen comprised of tamoxifen, doxorubicin, and PSC 833 increased ceramide levels 26-fold and brought cell viability to zero.

CONCLUSIONS. These results demonstrate that MDR modulators can be used separately, in combination, or in conjunction with chemotherapy at clinically relevant concentrations to manipulate cellular ceramide levels and restore sensitivity in the drug resistant setting. As such, this represents a new direction in the treatment of cancer. *Cancer* 1999;86:300-11. © 1999 American Cancer Society.

KEYWORDS: multidrug resistance, ceramide, breast carcinoma, tamoxifen, SDZ PSC 833.

Multidrug resistance (MDR) is a formidable roadblock to the effective treatment of cancer by conventional chemotherapy. Treatment in many instances is complicated by resistance phenomena. For example, despite the popularity of taxanes as antitumor agents, clinical resistance^{1,2} poses a threat to successful treatment. Complementary with efforts to design more efficacious antineoplastics is research on the development of agents to reverse MDR. These

agents, termed MDR modulators, although they are not chemotherapeutic drugs, per se, represent an exciting genre of therapeutics that, when used with chemotherapy, often restore sensitivity in an otherwise resistant setting.³

The greatly reduced sensitivity to anticancer drugs, a hallmark of MDR, often results from overexpression of P-glycoprotein (P-gp), a 170-kilodalton (kDa) plasma membrane protein that functions as a drug efflux pump.^{4,5} MDR also is caused by cellular increases in glutathione S-transferase⁶ and changes in the activity of topoisomerase II.⁷ A major challenge in cancer chemotherapy is to delineate the molecular mechanisms by which MDR modulators, e.g., tamoxifen, cyclosporine A, and SDZ PSC 833 (PSC 833) ([3'-keto-Bmt-1]-[Val-2]-cyclosporine), reverse drug resistance. These agents have been shown to bind directly to P-gp^{8,9} and thereby interfere with binding and export of anticancer drugs. Tamoxifen, an antiestrogen used in treatment of breast carcinoma long known for MDR modulatory properties,³ binds to P-gp,¹⁰ as does the nonimmunosuppressive cyclosporine A analog, PSC 833,¹¹ a potent drug-resistance modulator.¹² PSC 833 is more effective than verapamil and cyclosporine A in reversing MDR in vitro and in vivo.¹³

To provide insight for the development of more effective clinical agents and more effective drug regimens, we focused on elucidating the mechanisms of action of MDR modulators. We recently demonstrated an association between MDR and glucosylceramide¹⁴ and, in turn, showed that many of the MDR modulators, including tamoxifen and cyclosporine A, retard the conversion of ceramide to glucosylceramide in drug-resistant cancer cells.¹⁵ Glucosylceramides serve as precursors for synthesis of over 200 known glycosphingolipids. They are postulated to have a role in the regulation of cell proliferation.¹⁶⁻¹⁸ Whereas ceramide is a lipid messenger that mediates apoptosis,¹⁹ glucosylceramide has not been shown to regulate programmed cell death. We propose that the build-up of glucosylceramides is a molecular determinant of MDR, representing the enhanced capacity of some tumor cells to convert toxic ceramide to nontoxic glucosylceramide. We recently demonstrated that transfection to overexpress glucosylceramide synthase, the enzyme converting ceramide to glucosylceramide, confers resistance to doxorubicin and to ceramide in human breast carcinoma cells.²⁰ This clearly establishes a role for up-regulated ceramide metabolism in cell survival.

With ceramide now recognized as a cellular messenger of apoptosis and apoptosis now seen as an important element in the cytotoxic response to antineoplastics,²¹ the impact of MDR modulators on ceramide metabolism

takes on clinical relevance. We show here that PSC 833 has a profound effect on glycolipid metabolism. However, unlike the chemical analog, cyclosporine A, which inhibits cellular glucosylceramide formation,¹⁵ PSC 833 activates glucosylceramide and ceramide formation in MDR cancer cells. We show that the increase in glucosylceramide through glucosylceramide synthase is in response to the initial burst in ceramide formation caused by PSC 833. In addition, when PSC 833 is employed in combination with doxorubicin and tamoxifen, ceramide levels increased many fold over controls, and cell viability in the MDR model fell to zero. This interplay of chemotherapeutic drugs and drug resistance modulators, with associated synergistic impact on ceramide metabolism, provides a new direction for the treatment of cancer.

MATERIALS AND METHODS

Cells

Experiments were conducted using the human breast carcinoma cell lines MCF-7-AdrR (doxorubicin resistant) and wild-type MCF-7 (provided by Dr. Kenneth H. Cowan and Dr. Merrill E. Goldsmith, National Cancer Institute). Cells were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and antibiotics as described previously.¹⁵ Cultures were maintained in a humidified 5% CO₂ atmosphere incubator. Trypsin (0.05%) and ethylenediamine tetraacetic acid (EDTA) (0.5 mM) were used for subculture. Plastic tissue cultureware was from Costar (Cambridge, MA) (96-well plates) and Corning (Corning, NY) (6-well plates, 6-cm and 10-cm dishes, T-75 flasks).

Cell Radiolabeling and Lipid Analysis

To assess lipid metabolism, cells were grown in the presence of tritiated ceramide precursors, L-[³H]serine (20 Ci/mmol) (American Radiolabeled Chemicals, Inc., St. Louis, MO) and [9,10-³H]palmitic acid (50 Ci/mmol) (DuPont NEN, Boston, MA). Labeling media were prepared by adding microliter amounts of tritiated compounds (supplied in ethanol or sterile water) to medium containing 5% FBS. After the radiolabeling period, 0.1 mL aliquots of medium were removed and analyzed by liquid scintillation counting (LSC) to determine cellular uptake.¹⁵ Cell monolayers were then rinsed twice with cold phosphate-buffered saline. Ice-cold methanol containing 2% acetic acid was added, and cells were scraped free of the substratum using a plastic scraper. Cellular lipids were extracted by using the method of Bligh and Dyer.²² After centrifugation, the resulting organic lower phase was withdrawn, transferred to a glass vial, and evaporated to dryness under a stream of nitrogen.

Radioactivity in glucosylceramide was analyzed by

thin-layer chromatography (TLC) of total cell lipids using a solvent system containing chloroform/methanol/ammonium hydroxide (80:20:2, volume/volume). Silica Gel G plates were used (Analtech, Newark, DE). Migration of glucosylceramide was compared with commercial standards (glucocerebrosides, Gaucher's spleen) obtained from Matreya, Inc. (Pleasant Gap, PA), and lipid spots, after iodine vapor visualization, were scraped for tritium quantitation by LSC.^{11,15} [³H]Ceramide was resolved from other labeled lipids by TLC using a solvent system containing chloroform/acetic acid (90:10, volume/volume), and [³H]sphingomyelin was resolved by TLC in chloroform/methanol/acetic acid/water (60:30:7:3, volume/volume). Ceramide and sphingomyelin (brain-derived) were obtained from Avanti Polar Lipids (Alabaster, AL). Ceramide was analyzed by using an alternate method that consisted of subjecting an aliquot of the total cell lipid extract to mild alkaline hydrolysis (0.1 N KOH in methanol, 1 hour at 37°C) followed by reextraction.¹⁵ Ceramide was then resolved by TLC in a solvent system containing hexane/diethyl ether/formic acid (60:40:1, volume/volume). Both methods of ceramide analysis yielded similar results.

Cytotoxicity Assays

MCF-7-AdrR or MCF-7 cells, counted by hemocytometer, were seeded into 96-well plates (2000–2500 cells/well) in 0.1 mL RPMI-1640 medium containing 5% FBS. We do not use perimeter wells of the 96-well plates for cells; perimeter wells contained 0.2 mL water. Cells were cultured for 24 hours before addition of drugs. Drugs were dissolved in the appropriate vehicles (see below), diluted into 5% FBS-containing medium, and added to each well in a final volume of 0.1 mL. Cells were incubated at 37°C for the times indicated. The cytotoxic activity of a drug was determined by using the Promega Cell Titer 96 aqueous cell proliferation assay kit (Promega, Madison, WI). Each experimental point was performed in six replicates. Promega solution (20 μ L, not the suggested 40 μ L) was aliquoted to each well, and cells were placed at 37°C for 1–2 hours or until an optical density of 0.9–1.2 was obtained as the highest reading. Absorbance at 490 nm was recorded using an enzyme-linked immunosorbent assay plate reader (Molecular Devices, San Diego, CA).

Determination of Apoptosis

MCF-7-AdrR cells were seeded in 10-cm dishes in medium containing 5% FBS. After attachment, cells were treated with vehicle (control), 5.0 μ M PSC 833, 10 μ M tamoxifen, or PSC 833 plus tamoxifen for a total of 48 hours. Cells were then harvested by trypsin-EDTA, isolated by centrifugation, and incubated with diges-

tion buffer (100 mM NaCl, 25 mM EDTA, 10 mM Tris-HCl, 0.5% sodium dodecylsulfate, 0.3 mg/mL proteinase K, pH 8.0) at 45°C for 18 hours. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1, volume/volume) and precipitated in a 1:3 volume of 7.5 M ammonium acetate and 2 volumes of 100% ethanol at –20°C overnight. The preparation was centrifuged for 20 minutes at $\times 10,000g$ at 4°C. RNA was digested in buffer containing 10 mM Tris-HCl, 0.1 mM EDTA, 0.1% sodium dodecylsulfate, and 100 units/mL RNase at 37°C for 2 hours. Reextracted DNA (2.0 μ g) was analyzed by electrophoresis on a 2% agarose gel in buffer (40 mM Tris-acetate, 1.0 mM EDTA, pH 8.3). DNA fragments were visualized with ethidium bromide under ultraviolet light.

Drugs and Vehicles

Doxorubicin (Sigma Chemical Co., St. Louis, MO) was prepared in sterile water at a final concentration of 1.0 mM. Tamoxifen (free base; Sigma Chemical Co.) was prepared as a 20-mM stock solution in acetone. PSC 833 and cyclosporine A (Sandimmune), were provided by Novartis (East Hanover, NJ). Stock solutions (10 mM) of PSC 833 and cyclosporine A were prepared in ethanol. Fumonisin B₁ (FB₁) was purchased from Sigma Chemical Co., and stock solutions (5 mM) were prepared in phosphate-buffered saline. All stock solutions were prepared in 1-dram glass vials with Teflon-lined screw caps and stored at –20°C. Culture media containing drugs were prepared just prior to use. Vehicles were present in control (minus drug) cultures at final concentrations of 0.01–0.1%.

RESULTS

PSC 833 and Cyclosporine A have Opposing Effects on Glycolipid Metabolism

In previous work, we demonstrated that several structurally dissimilar MDR modulators retard glucosylceramide formation by interference with ceramide glycosylation.^{15,23} These findings may have clinical relevance in view of studies showing that ceramide elicits apoptosis.^{24,25} Because earlier work from our laboratory showed that glucosylceramide characteristically is elevated in MDR cancer cells as opposed to chemosensitive counterparts,¹⁴ we have endeavored to pinpoint the role of glucosylceramide in drug-resistance biology. In this paper, using the cyclosporine A analog, PCS 833, we demonstrate a commonality between glycolipids and the action of MDR modulators; however, unlike our previous work showing inhibition of glucosylceramide formation,^{15,23} it is shown here that PSC 833 activates glucosylceramide formation. Preliminary experiments using TLC autoradiography showed that glu-

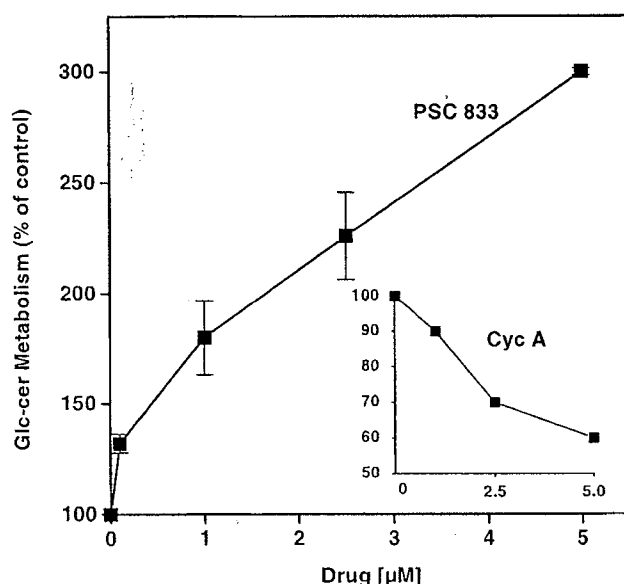


FIGURE 1. Dose response effect of cyclosporines on glucosylceramide metabolism in doxorubicin-resistant human breast carcinoma cells (MCF-7-AdrR). Cells were seeded into six-well plates. At 60% confluence, they were treated with either cyclosporine A (Cyc A) or PSC 833 at the concentrations indicated for 1 hour before the addition of [3 H]palmitic acid (1.0 μ Ci/mL medium) for an additional 23 hours. Total lipids were extracted, and glucosylceramide was quantitated by thin-layer chromatography and liquid scintillation counting, as described in Materials and Methods. Glc-cer: glucosylceramide.

cosylceramide was nearly depleted in cells exposed to cyclosporine A; however, PSC 833 caused glucosylceramide levels to increase markedly. The opposing effects of the cyclosporines on glucosylceramide metabolism are seen readily in a dose response experiment (Fig. 1). Whereas increasing the concentration of cyclosporine A inhibited glucosylceramide formation (Fig. 1, inset), increasing the concentration of PSC 833 resulted in enhanced glucosylceramide formation (Fig. 1). Activation of glucosylceramide formation was apparent at levels of PSC 833 as low as 0.1 μ M.

Because ceramide plays a key role in signaling events leading to apoptosis, it was of interest to determine whether the alteration in glucosylceramide metabolism elicited by PSC 833 was linked to changes in the metabolism of ceramide. Initial experiments showed that PSC 833 affected an increase in cellular ceramide synthesis that preceded the increase in glucosylceramide. The experiments also showed that enhanced formation of ceramide and glucosylceramide was dose dependent with respect to PSC 833 over a range of 0.1–5.0 μ M. The influence of PSC 833 exposure time on the metabolism of ceramide, glucosylceramide, and sphingomyelin is shown in Figure 2. En-

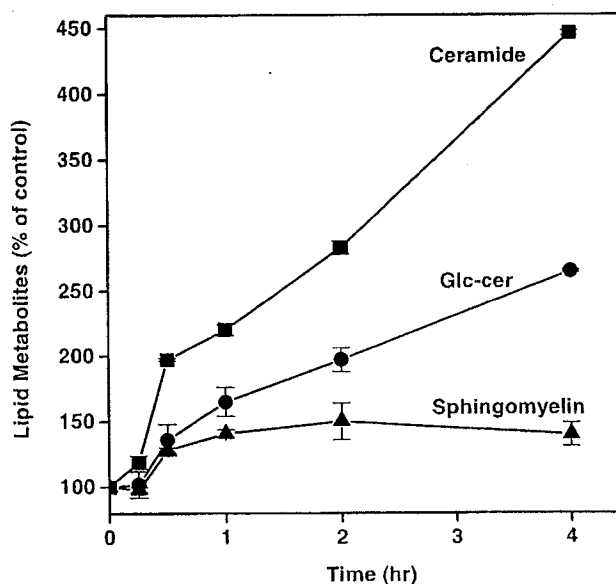


FIGURE 2. Time course of the effect of PSC 833 on the metabolism of ceramide and sphingolipids. Doxorubicin-resistant human breast carcinoma (MCF-7-AdrR) cells were seeded into six-well plates. At approximately 70% confluence, [3 H]palmitic acid (1.0 μ Ci/mL medium) and PSC 833 (5.0 μ M) were added simultaneously for the times indicated. Lipids were extracted, and ceramide, glucosylceramide (Glc-cer), and sphingomyelin were analyzed by thin-layer chromatography and liquid scintillation counting, as described in Materials and Methods.

hanced formation of ceramide was discernible as early as 30 minutes after cells were treated with PSC 833, and, at all times thereafter, rates of ceramide formation foreran rates of glucosylceramide formation. Sphingomyelin radioactivity also increased in response to PSC 833, and, at 2 hours, levels were 50% above control values.

Metabolic Pathway of Ceramide Formation

The small increase in sphingomyelin shown in Figure 2 suggests that PSC 833 does not elicit ceramide formation through the action of sphingomyelinase. If PSC 833 promoted ceramide formation through activation of sphingomyelinase, which cleaves sphingomyelin into ceramide and phosphorylcholine, then radioactivity in sphingomyelin would be expected to decrease. To delineate more fully the metabolic pathway of ceramide formation, FB₁, an inhibitor of ceramide synthase, was employed. Cells were exposed to PSC 833 (5 μ M) in the absence or presence of FB₁ (100 μ M), in medium containing [3 H]palmitic acid. After 2 hours, ceramide radioactivity measured 42,795 \pm 3168 cpm and 11,861 \pm 549 cpm in the absence and presence of FB₁, respectively. This represents an approximate 70% depression in PSC 833-induced ceramide

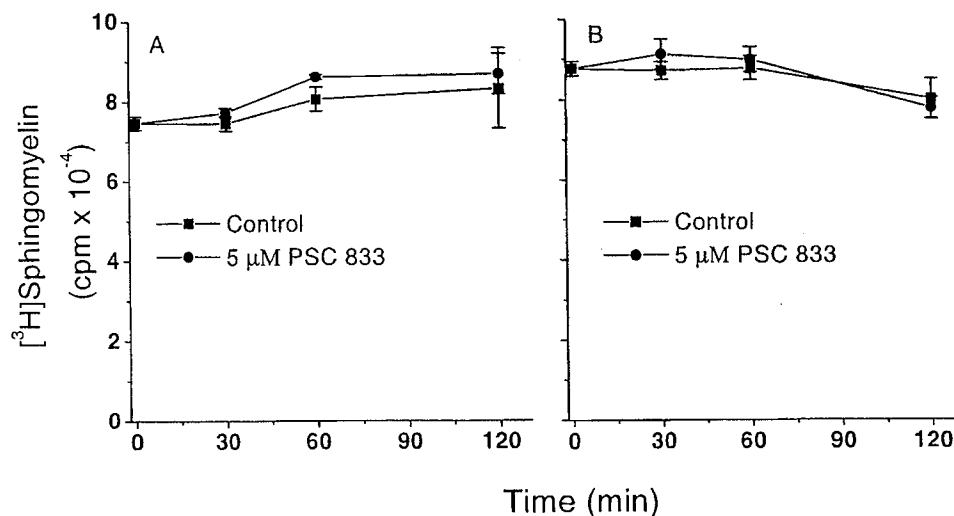


FIGURE 3. The influence of PSC 833 on sphingomyelin metabolism in doxorubicin-resistant human breast carcinoma (MCF-7-AdrR) cells. Cells in six-well plates at approximately 50% confluence were cultured with $[^3\text{H}]$ palmitic acid (1.0 $\mu\text{Ci}/\text{mL}$ medium) for 24 hours (A) or with $[^3\text{H}]$ serine (4.0 $\mu\text{Ci}/\text{mL}$ medium) for 48 hours (B). After removal of labeling medium, cultures were rinsed three times in aged medium (medium minus radiolabel conditioned at 37°C and CO_2 equilibrated for 24 hours or 48 hours) and then treated in the absence or presence of PSC 833 (5.0 μM) in aged medium for the times indicated. Cell lipids were extracted, and $[^3\text{H}]$ sphingomyelin was analyzed by thin-layer chromatography and liquid scintillation counting. The cpm in sphingomyelin represents counts per 500,000 cpm total lipid tritium.

formation when FB_1 is present. Cell prelabeling experiments also were conducted to assess the influence of PSC 833 on the metabolic fate of sphingomyelin. These experiments, using $[^3\text{H}]$ palmitic acid (Fig. 3A) and $[^3\text{H}]$ serine (Fig. 3B) to radiolabel sphingomyelin pools, show that the decay of $[^3\text{H}]$ sphingomyelin was identical in the absence and presence of PSC 833. In summary, the inclusion of a ceramide synthase inhibitor blocked PSC 833-induced ceramide generation, and PSC 833 did not accelerate the disappearance of cellular sphingomyelin. These data strongly imply that PSC 833 activation of ceramide formation is through a de novo ceramide synthase route and not by enzymatic hydrolysis of sphingomyelin.

PSC 833 Enhances Doxorubicin Toxicity in MDR Breast Carcinoma Cells

Because PSC 833 alone increases cellular ceramide levels, preliminary experiments were conducted to define the influence of PSC 833 on cell viability. Comparing drug-sensitive MCF-7 wild type with MDR (MCF-7-AdrR) cells, the data in Figure 4 show that MDR cells are more resistant to PSC 833 toxicity. At higher concentrations (10 μM), MDR cell survival was 65%; whereas MCF-7 cells were more sensitive to PSC 833 (20% survival at 10 μM). The differential sensitivity to PSC 833 may be linked to differences in the ceramide metabolizing capacities of the two cell lines (see Discussion). The influence of PSC 833 on doxorubicin toxicity was then assessed. Figure 5 demonstrates that MCF-7-AdrR cells were essentially refrac-

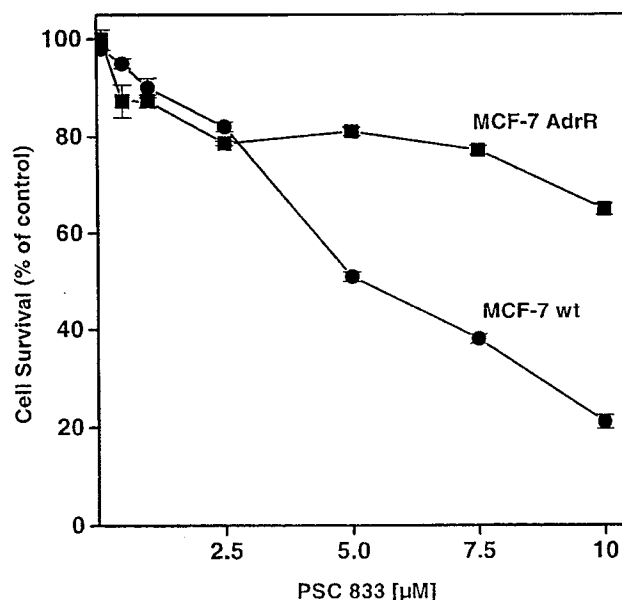


FIGURE 4. Effect of PSC 833 on cell viability in chemosensitive and chemoresistant models. MCF-7 wild-type (wt; chemosensitive) and MCF-7-AdrR cells were seeded into 96-well plates (2000 cells/well) and treated the following day with PSC 833 at the concentrations indicated. After 4 days, cell survival was measured using the cell proliferation assay reagents described in Materials and Methods. Each point represents the average of six replicate assays.

tory to doxorubicin treatment. Over a concentration range of 0.1–1.0 μM , cell survival was within 80–95% of control values. PSC 833 at a concentration of 0.5 μM elicited only negligible toxicity; however, when PSC 833 was maintained at 0.5 μM and escalating doses of

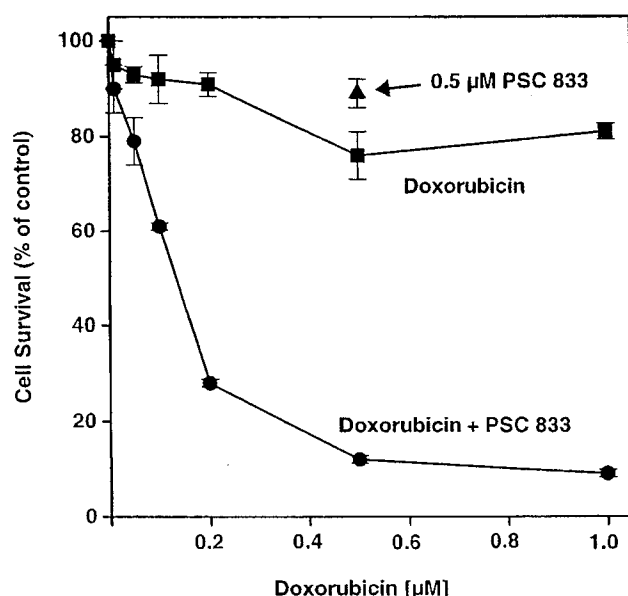


FIGURE 5. Modulation of doxorubicin resistance by PSC 833. MCF-7-AdrR cells were seeded into 96-well plates (2500 cells/well) and treated 24 hours later with vehicle (control), 0.5 μ M PSC 833 only, doxorubicin at increasing concentrations, or 0.5 μ M PSC 833 plus the doxorubicin concentrations indicated. After a 5-day incubation in the presence of drugs, cell viability was determined. Each experimental point represents the average of six replicates.

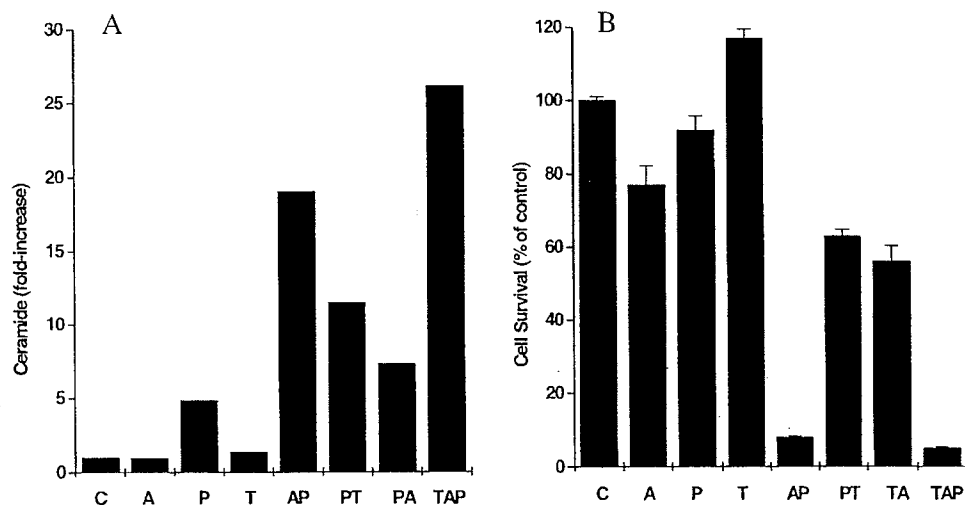
doxorubicin were administered, cell viability dropped precipitously. At 0.2 μ M doxorubicin (Fig. 5, upper curve), cell survival (90%) was on a parallel with survival of cells exposed to 0.5 μ M PSC 833 alone. When the two agents were mixed, cell survival fell to 28% (Fig. 5, lower curve).

Combination Therapeutics: Influence on Ceramide Metabolism and Cell Viability

Because ceramide is potentially toxic through signaling events that lead to programmed cell death, it was important to evaluate ceramide metabolism in a setting where PSC 833 is used in combination with antineoplastic agents. We also included the antiestrogen, tamoxifen, a drug-resistance modulator that blocks ceramide glycosylation.^{15,23} In previous studies, tamoxifen inhibition of glucosylceramide synthesis in MCF-7-AdrR cells had an EC_{50} of 1.0 μ M, and toxic responses to tamoxifen were not observed with levels as high as 5.0 μ M. In addition, doxorubicin at 2.5 μ M is only slightly cytotoxic.¹⁵ PSC 833 at a concentration of 5.0 μ M also has little negative impact on MCF-7-AdrR cell survival (Fig. 4). Therefore, synergy experiments for drug combinations were carried out at the above doses. The data in Figure 6A reveal that combination therapies have a marked impact on cellular ceramide production. In

cells exposed to doxorubicin alone, ceramide formation was not altered. In contrast, PSC 833 caused a nearly 5-fold increase in ceramide levels. Tamoxifen produced a slight 1.3-fold increase in ceramide. When doxorubicin and PSC 833 were coadministered, cellular ceramide levels rose 19-fold compared with controls. Likewise, with combinations of PSC 833 and tamoxifen and tamoxifen plus doxorubicin, ceramide levels increased 11.5-fold and 7.3-fold over controls, respectively. The tamoxifen (T), doxorubicin (A), PSC 833 (P) (TAP) regimen produced a 26-fold increase in cellular ceramide. Cell viability was then evaluated among the various drug combinations studied. The data show that drug combinations eliciting the greatest elevation in ceramide were the most cytotoxic (Fig. 6B). Doxorubicin was slightly cytotoxic in the MDR model, with growth inhibition of 25%. PSC 833 alone was nearly without influence (8% kill), and tamoxifen produced a moderate 20% growth stimulation. Combinations of PSC 833 plus tamoxifen and tamoxifen plus doxorubicin reduced cell survival to approximately 60%. It is noteworthy that PSC 833 and tamoxifen, neither of which is a cytotoxic chemotherapeutic agent, had an overall positive impact of 12% on cell growth (Fig. 6B, P, T); however, the mixture was cytotoxic (Fig. 6B, PT) and stimulated ceramide formation (Fig. 6A, PT). The doxorubicin-containing mixtures, doxorubicin plus PSC 833 (AP) and TAP, brought cell viability to nearly zero (Fig. 6B). Lipid metabolism studies were conducted after 24 hours of exposure to drug, whereas cytotoxicity was evaluated at 3–4 days, demonstrating that ceramide increased prior to cytotoxic responses. This suggests that upstream ceramide signals downstream apoptosis.

Steady-state radiolabeling of cells with long chain fatty acids can be achieved within 24 hours. Therefore, the percent incorporation of tritium into complex cellular lipids is reflective of actual mass changes in the lipids. In the experiment shown in Figure 6, the TAP regimen elicited a 26-fold increase in [3 H]ceramide levels. Using total lipid radioactivity, it was calculated that ceramide accounted for 0.5% of total lipid tritium in control cultures and 14% of total lipid tritium in TAP-treated cultures, an increase of 28-fold. Although this illustrates the impact of TAP on radioactive ceramide levels, we sought to measure ceramide increases on a mass basis. The chromatogram in Figure 7 shows that ceramide was nearly undetectable in untreated controls, which would be expected for this intermediate of complex glycosphingolipids. However, in cells treated with TAP, ceramide mass increased strikingly (Fig. 7, right lane) and showed the doublet characteristic of mixed chain-length species.



This chromatogram demonstrates that the drugs promote an actual increase in the mass of ceramide, exemplifying the cytotoxic potential of such regimens.

Induction of Apoptosis

It was of interest to determine whether drugs that are not known to be cytotoxic yet synergize to enhance ceramide formation would cause apoptosis. In combination, PSC 833 and tamoxifen caused a >11-fold increase in cellular ceramide levels (Fig. 6A). The data in Figure 8 demonstrate that the PSC 833-tamoxifen combination elicited DNA fragmentation (Fig. 8, lane 4), a hallmark of apoptosis, whereas neither PSC 833 nor tamoxifen (Fig. 8, lanes 2 and 3, respectively) caused DNA damage. The apoptosis experiment was conducted using tamoxifen at 10 μ M; therefore, the cell survival data shown in Figure 6B and DNA fragmentation of Figure 8 are not directly comparable. It is important, however, that, separately, PSC 833 and tamoxifen are neither toxic nor do these agents potentiate DNA fragmentation at the concentrations tested in Figure 8.

FIGURE 6. Effect of combination drug treatment on ceramide metabolism and viability in multidrug resistant (MDR) cells. (A) Ceramide metabolism. Doxorubicin-resistant human breast carcinoma (MCF-7-AdrR) cells were seeded into six-well plates. At 60–70% confluence, cells were treated with vehicle (C; control), doxorubicin (A; 2.5 μ M), PSC 833 (P; 5.0 μ M), tamoxifen (T; 5.0 μ M), or the combinations indicated (AP, PT, PA, TAP) for 24 hours in the presence of [3 H]palmitic acid (1.0 μ Ci/mL culture medium). Lipids were extracted, and ceramide was analyzed by thin-layer chromatography and liquid scintillation counting. Data points represent the average of triplicate cultures. The experiment was repeated with similar results. (B) Effect of combination drug treatment on cell survival. MCF-7-AdrR cells were seeded into 96-well plates at 2000 cells/well and treated the following day with the indicated drugs: control (C; vehicle), doxorubicin (A; 2.5 μ M), PSC 833 (P; 5.0 μ M), tamoxifen (T; 5.0 μ M), or the combinations shown (AP, TP, TA, TAP). After 3 days of exposure, cell viability was evaluated.

MDR Modulation and Ceramide Metabolism at Clinically Relevant Doses

The doxorubicin-PSC 833 regimen markedly increased cellular ceramide and elicited a cytotoxic response (Fig. 6). To determine whether ceramide metabolism is influenced by drug doses that are more in line with reversal of resistance, as depicted in Figure 5, MCF-7-AdrR cells were exposed to a low dose regimen. Figure 9 shows that simultaneous exposure to PSC 833 (0.5 μ M) and doxorubicin (0.2 μ M) produced a synergistic increase in cellular ceramide. Whereas PSC 833 and doxorubicin alone caused 1.3-fold and 1.4-fold increases in ceramide, respectively, the mixture elicited a >2.5-fold increase in ceramide. These data show that doses as high as those used for the experiment in Figure 6 are not required to elicit cell responses, thus indicating the need for more extensive studies on dosing and synergy.

DISCUSSION

Multidrug resistance is a major obstacle to the successful treatment of cancer. Consequently, agents that modulate MDR, such as verapamil and SR33557,^{3,26}

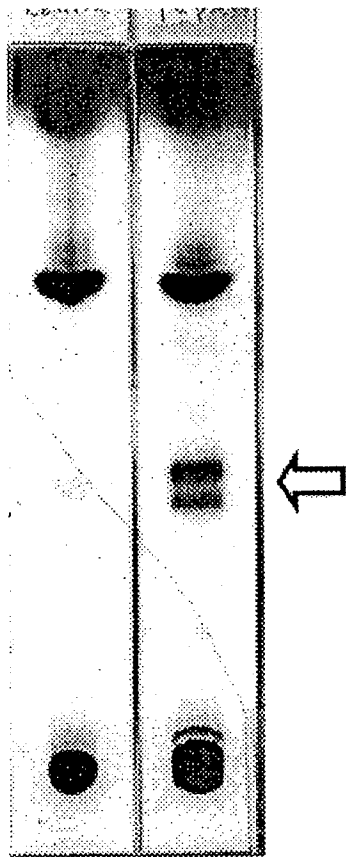


FIGURE 7. Influence of combination chemotherapy on ceramide mass levels in MCF-7-AdrR cells. Cells seeded into 10-cm dishes were grown to 70–80% confluence. Fresh medium containing 5% fetal bovine serum and drugs was then added for 24 hours. The TAP-treated cultures contained tamoxifen (T; 5.0 μ M), doxorubicin (A; 2.5 μ M), and PSC 833 (P; 5.0 μ M). Total cell lipids were extracted, and 200 μ g total lipid was spotted onto thin-layer chromatography plates. The chromatogram was developed in chloroform/acetic acid (90:10, volume/volume), air dried, sprayed with 30% H_2SO_4 , and charred in an oven at 180°C for 20 minutes. Left lane: control cells; right lane: cells treated with TAP. The arrow indicates ceramide.

the antiestrogens tamoxifen and toremifene,^{3,27,28} phenothiazines,^{3,29} quinacrine and quinine,^{3,30} amiodarone,³¹ cyclosporine A,³² GF120918,³³ and VX-710,³⁴ have been the object of much study. MDR in many instances is mediated through overexpression of P-gp, and this, in turn, is associated with decreased drug accumulation. P-gp can be expressed either constitutively, as with colorectal and renal carcinomas, or by an acquired mechanism, as in leukemia, breast, and ovarian carcinomas. A myriad of agents, including some of those listed above, has been shown to reverse drug resistance by a P-gp-mediated mechanism believed to involve direct binding of the agent. In a recent study of MDR modulators, we showed that cyclosporine A, tamoxifen, and verapamil all blocked

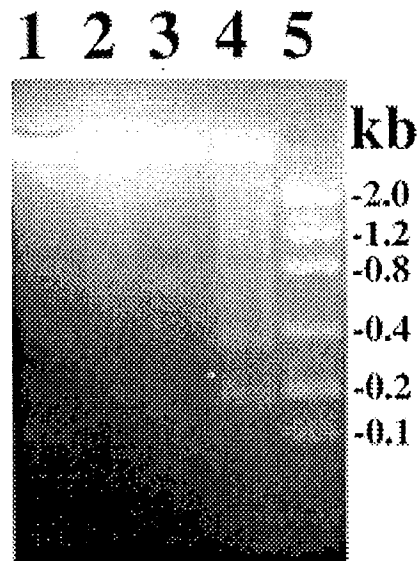


FIGURE 8. Influence of PSC 833 and tamoxifen on DNA integrity in MCF-7-AdrR cells. Cells were seeded in medium containing 2.5% fetal bovine serum and treated with vehicle (control), 5.0 μ M PSC 833, 10 μ M tamoxifen, or PSC 833 plus tamoxifen for 48 hours. Cellular DNA was analyzed on 2% agarose gels, as detailed in Materials and Methods. Lane 1: control; lane 2: PSC 833; lane 3: tamoxifen; lane 4: PSC 833 plus tamoxifen; lane 5: DNA, 200–2000 base pair commercial standard.

glycolipid metabolism effectively in doxorubicin-resistant cells.¹⁵ This was accompanied by increased sensitivity to chemotherapy. These lipid responses suggest that a biological mechanism in addition to the suppression of drug efflux is common to the action of some chemosensitizers.

In an effort to elucidate the role of glycolipids in drug-resistance modulation, we initiated work with PSC 833. Here, we show that PSC 833 is a strong agonist of glycolipid metabolism. Although this is in marked contrast with the inhibition of glycolipid metabolism elicited by cyclosporine A, tamoxifen, and verapamil,¹⁵ nevertheless, the MDR modulators we have tested evoke a common influence on the glycolipid machinery of the cell. The increase in glucosylceramide in response to PSC 833 exposure occurred through an upstream influence on ceramide as opposed to a downstream inhibitory effect on the degradative enzyme, glucocerebrosidase. Experiments showed that ceramide formation was activated by PSC 833. The pattern depicted in Figure 2 is suggestive of a precursor-product relation wherein PSC 833 elicits an increase in ceramide, which is then converted through glycosylation to glucosylceramide: hence, the increase in glucosylceramide formation caused by PSC 833. When cells are challenged with PSC 833, ceramide can rise to toxic levels. A proven survival pathway that we

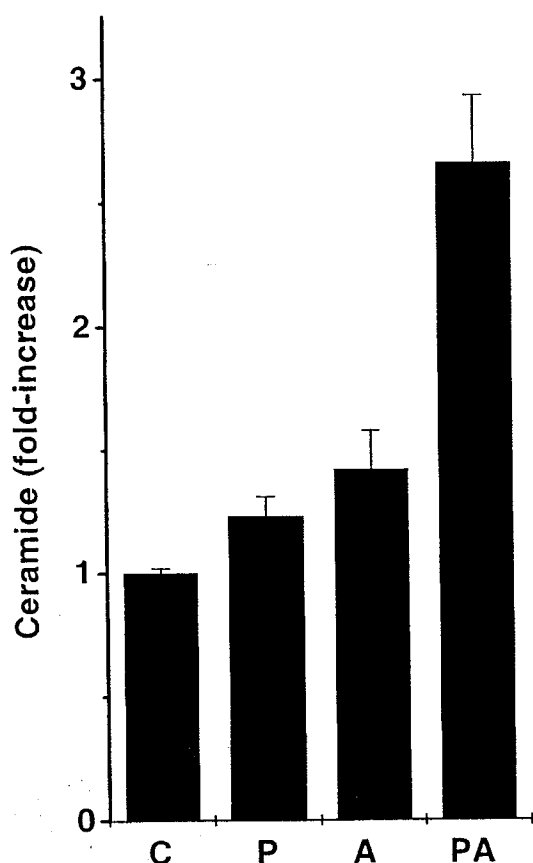


FIGURE 9. Influence of low dose chemotherapy on ceramide levels in MCF-7-AdrR cells. Cells were seeded in six-well plates and were treated the following day with vehicle (C; control), PSC 833 (P; 0.5 μ M), doxorubicin (A; 0.2 μ M), or a mixture of P and A for 4 days in medium containing [3 H]palmitic acid (1.0 μ Ci/mL). Ceramide was resolved by thin layer chromatography, and tritium was analyzed by liquid scintillation counting. The fold-increase in ceramide is based on radioactivity in ceramide per 500,000 cpm total lipid tritium, and each data point represents the mean \pm standard deviation ($n = 3$).

have shown through gene transfection studies²⁰ is to lessen cellular ceramide levels through up-regulated conversion of ceramide to glucosylceramide. Ceramide also can be converted to sphingomyelin by sphingomyelin synthase. Therefore, the increase in sphingomyelin shown in Figure 2 likely is due to further effort at the cellular level to reduce the amount of endogenous ceramide. It should be mentioned, however, that the cell will put limits on the amount of ceramide that is shuttled into sphingomyelin, because sphingomyelin is an important membrane building element. Over-synthesis of sphingomyelin likely would result in cell damage, whereas cells can accumulate glucosylceramide with little consequence.¹⁴ The scheme shown in Figure 10 illustrates ceramide synthesis and metabolism and depicts the sites at which the various drugs act. The figure shows how

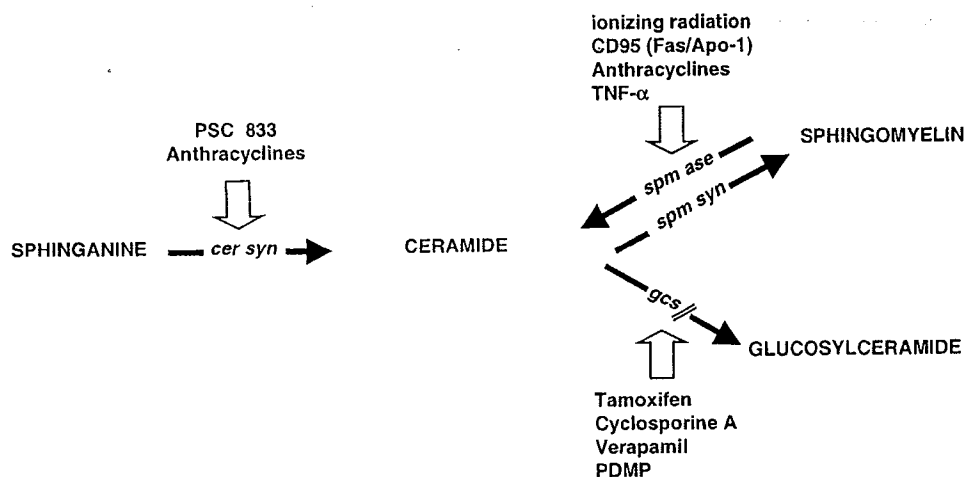
drugs from the different categories may synergize to affect enhanced cell killing. For example, PSC 833, which promotes ceramide synthesis *de novo*, combined with an anthracycline, which promotes ceramide generation through a sphingomyelinase route, in the presence of tamoxifen, a glucosylceramide synthesis inhibitor, raises ceramide to toxic levels.

The pathway of PSC 833-activated ceramide formation appears, from our results, to be through ceramide synthesis as opposed to sphingomyelin degradation. This pathway would include, in addition to ceramide synthase, activated formation of ceramide precursors, such as sphinganine, which is formed by reduction of 3-ketodihydrosphingosine with reducing equivalents from nicotinamide adenine dinucleotide phosphate. FB₁, an inhibitor of ceramide synthase, blocked PSC 833-induced ceramide formation in MCF-7-AdrR cells. Furthermore, experiments utilizing cells in which the sphingomyelin pools had been preradiolabeled to equilibrium showed that PSC 833 did not influence sphingomyelin decay (Fig. 5). Therefore, the PSC 833 ceramide pathway is similar to daunorubicin-induced ceramide formation through ceramide synthase in P388 murine leukemia cells³⁵ but differs from ceramide generation through sphingomyelinase elicited by tumor necrosis factor- α , ionizing radiation, or Fas/Apo-1.³⁶⁻³⁸ Regarding daunorubicin activity, a more recent report showing ceramide generation in HL-60 and U937 human leukemia cells through sphingomyelinase,³⁹ and not ceramide synthase,³⁵ demonstrated tumor cell type specificity for ceramide formation. Concerning the role of P-gp, the MCF-7-AdrR cells used in the current study are rich in P-gp, whereas the MCF-7 parent cell line is void by comparison (Western blot analysis; data not shown). Although P-gp acts as a lipid translocase with broad specificity,⁴⁰ and PSC 833 binds to P-gp,¹¹ PSC 833 has been shown to activate ceramide formation in a P-gp-deficient model.⁴¹ This suggests that P-gp is not involved. It is possible that PSC 833 modifies transport of ceramide precursors at the level of the Golgi⁴², independent of P-gp.

The finding that PSC 833 causes elevation of cellular ceramide is significant in view of findings that link ceramide with the induction of apoptosis.^{24,25} The structure-activity correlation for activated formation of ceramide by PSC 833 must be stringent, because the structures of cyclosporine A and PSC 833 are very similar.¹¹ In PSC 833, the β -hydroxy amide of cyclosporine A is replaced by a β -keto amide, and one ethyl group is replaced by an isopropyl function. Cyclosporine A inhibits glucosylceramide formation (Fig. 1) and has no influence on ceramide generation.⁴¹

MDR modulation by PSC 833 results from an in-

FIGURE 10. Ceramide metabolism and sites of drug interaction. Agents listed on the left have been shown to increase ceramide synthesis by a de novo route, for example, PSC 833, doxorubicin, and daunorubicin. Agents on the right top enhance ceramide formation by activation of sphingomyelinase. The agents listed on the right bottom block ceramide glycosylation. cer syn: ceramide synthase; spm ase: sphingomyelinase; spm syn: sphingomyelin synthase; gcs: glucosylceramide synthase; PDMP: 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; TNF- α : tumor necrosis factor alpha.¹⁸



teraction with P-gp^{11,12} whereby enhanced toxicity to anticancer agents is achieved by increasing drug accumulation through inhibition of efflux. A comparison of cyclosporine A and PSC 833 shows that 1) the agents are only slightly different chemically; 2) PSC 833 is more potent in reversing drug resistance than cyclosporine A,^{12,43} although a recent study showed that cyclosporine A was superior to PSC 833 for enhancement of VP-16 toxicity in a murine tumor model;⁴⁴ 3) both agents bind P-gp, although PSC 833 is not transported like cyclosporine A;¹¹ and 4) PSC 833 elicits ceramide formation, whereas cyclosporine A does not. Both drugs are intended for use in the adjuvant setting administered as a codrug with chemotherapy. Therefore, to assess the potential role of ceramide in the cytotoxic response, the influence of PSC 833 on ceramide metabolism was investigated in drug-combination studies. The data demonstrate (Fig. 6) that ceramide generation is enhanced markedly by combining PSC 833 with other agents, and, in the instance of PSC 833 plus doxorubicin, ceramide levels in treated cells increased 19-fold over controls. Of particular relevance clinically are the data showing that low dose PSC 833, when combined with doxorubicin, caused a synergistic increase in cellular ceramide. A Phase I study in multiple myeloma showed PSC 833 peak blood levels in the range of 0.9–2.2 μ M, and in vitro studies showed that 2 μ M PSC 833 elicited rhodamine retention.⁴⁵ Work in progress using drug-sensitive KB-3-1 cells, a human epidermoid carcinoma cell line, shows that a PSC 833/vinblastine regimen is synergistic for ceramide formation and is more toxic than single-agent administration (unpublished data). These experiments illustrate that cytotoxicity increases in the absence of MDR using PSC 833, a P-gp modulator. Such findings may have important implications for therapeutic design.

In light of recent discoveries showing that tamoxifen inhibits ceramide glycosylation,^{15,23} the effect of tamoxifen on MDR reversal in the current study is noteworthy. Tamoxifen influenced neither ceramide formation nor cell viability (Fig. 6). PSC 833 caused a nearly 5-fold increase in ceramide; however, cytotoxicity remained low. Coadministration of tamoxifen and PSC 833 had a synergistic impact on ceramide levels (>11-fold over controls). Coadministration of tamoxifen and PSC 833 also resulted in apoptosis. Therefore, tamoxifen and PSC 833, although they are not chemotherapy drugs per se, were cytotoxic when combined. The MDR modulatory capacity of tamoxifen and other synthetic antiestrogens has long been known.^{27,28,46} Tamoxifen is a component of the Dartmouth regimen used to treat advanced stage melanoma,⁴⁷ and tamoxifen has been used in the treatment of pancreatic carcinoma⁴⁸ and malignant gliomas.⁴⁹ This intriguing property of tamoxifen, which is independent of estrogen receptor status, may be related to synergy that leads to enhanced ceramide generation. Bose et al.³⁵ have shown that daunorubicin increases ceramide levels and elicits apoptosis in leukemia cells. In our studies, we show that MDR cells rapidly convert ceramide to glucosylceramide.^{14,50} It is possible that tamoxifen intercedes at this biochemical junction and effectively blocks ceramide conversion to glucosylceramide.^{15,23} Although studies have emphasized that binding of tamoxifen to P-gp is key in MDR modulation,¹⁰ the involvement of ceramide may play a significant role.

In this study, we have shown that the MDR modulator PSC 833 has a marked impact on cellular ceramide formation, and, in combination therapies using doxorubicin and/or tamoxifen, there is prominent synergy and cytotoxicity. In a comparison of wild-type and MDR cells, it also was shown that MDR cells were

more resistant to PSC 833 (Fig. 4). This may be due to the capacity of MDR cells to glycosylate ceramide^{14,15,50} that is generated in response to PSC 833 treatment. The current information on ceramide signaling and the role of apoptosis as a cell mechanism important for cytotoxic response to antineoplastics^{21,36-39} highlights the need for further investigation along these lines. The ultimate relevance of these in vitro studies to clinical outcome remains to be established. Experiments with normal cells and in vivo studies are indicated. Our studies suggest that the MDR phenotype and/or the expression of P-gp are not requisites for PSC 833 activity,⁴¹ and work with transfection of MCF-7 wild-type cells with the enzyme glucosylceramide synthase²⁰ shows that the MDR phenotype can be governed by cellular capacity to metabolize ceramide. Overall, the results of our studies imply that PSC 833 has a bimodal mechanism of action for enhancement of chemotherapy sensitivity in P-gp positive systems. The profound impact on ceramide metabolism, coupled with classical inhibition of drug efflux, makes PSC 833 an intriguing agent for further study. Understanding how MDR reversing agents modulate pharmacokinetics and toxicity of chemotherapy drugs is essential for the design of new agents to treat cancer.

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